UNIVERSITY OF ALBERTA

THE DEVELOPMENT, CHARACTERIZATION AND WOUND HEALING IN THE KERATIN-14-PROMOTED TRANSFORMING GROWTH FACTOR-β1 TRANSGENIC MOUSE

By

TEDDY CHAN

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE**

in

EXPERIMENTAL SURGERY

Department of Surgery

Edmonton, Alberta
Spring 2000.

National Library of Canada

Acquisitions and Bibliographic Services

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque nationale du Canada

Acquisitions et services bibliographiques

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence

Our file Notre référence

The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

L'auteur conserve la propriété du droit d'auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-60108-0

Canada

UNIVERSITY OF ALBERTA

LIBRARY RELEASE FORM

NAME OF AUTHOR: **Teddy Chan**

TITLE OF THESIS: **The Development, Characterization and Wound Healing of the Keratin-14-Promoted Transforming Growth Factor-β1 Transgenic Mouse**

DEGREE: **Master of Science**

YEAR THIS DEGREE GRANTED: **2000**

Permission is hereby granted to the University of Alberta Library to reproduce single copies of this thesis and to lend or sell such copies for private, scholarly or research purposes only.

The author reserves all other publication and other rights in association with the copyright in the thesis, and except as herein before provided, neither the thesis nor any portion thereof may be printed or otherwise reproduced in any material whatever without the author's prior written permission.

15912-89A Street,
Edmonton, AB
T5Z 3E2

Date: January, 27, 2000

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **the Development, Characterization, and Wound Healing of the Keratin-14-Promoted Transforming Growth Factor-β1 Transgenic Mouse** submitted by **Teddy Chan** in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE** in **EXPERIMENTAL SURGERY.**

Dr. Edward E. Tredget

Dr. Aziz Ghahary

Dr. Paul G. Scott

Dr. Peter Dickie

DATE: January 27, 2000

ABSTRACT

Transforming growth factor-beta 1 (TGF-β1) stimulates extracellular matrix synthesis at the same time inhibiting matrix degradation (Gleizes et al, 1997; Zhang et al, 1995). The study of a genetically manipulated animal, which produces excessive TGF-β in the skin, would be useful to evaluate its role in the epidermis and may help to further understand the development of fibrosis.

We developed a transgenic mouse which specifically targeted human TGF-β1 to the skin using a keratin 14 promoter and was subsequently bred into homozygous and heterozygous lines. The transgene was shown to be preferentially expressed in the skin without any detectable systemic effects. The only phenotypic difference noted was signs of 'scruffiness' in only the homozygous lines of mice.

Wound healing in these animals showed increased levels of TGF-β1 protein in the transgenic animals which would explain the delayed re-epithelialization observed. The less than dramatic effects in the dermis post-wounding are attributed to the lack of sufficient activation of the latent TGF-β1 in these animals.

In conclusion, we have developed a transgenic mouse that will be useful in future studies and have shown that excessive TGF-β1 from keratinocytes can impede wound healing.

Acknowledgments

I would like to thank the members of my supervisory committee, Drs. Tredget, Ghahary, Scott and Dickie for their support and invaluable guidance throughout this thesis.

A special thanks to the following people for their expertise, without which this project would have encountered even more obstacles: Dr. Peter Dickie (University of Alberta Transgenic Mouse Facility), whom not only served on my committee, but performed the microinjections to create our transgenic mouse; Patricia Murray (Nucleic Acid Research Center, University of Alberta) sequenced my PCR products; and Liju Yang (Plastic Surgery Research Lab) whose expertise in the PAI/L assay provided me with important information that it would otherwise be difficult to obtain.

I would also like to extend my thanks to my immediate and extended family of friends and co-workers in the lab. You all helped me keep my sanity by putting up with my insanity.

Last but definitely not the least, a special thank you to Amy Kwan for your patience and understanding. Whose words of encouragement kept me going through the long hours and whose moral support never wavered.

TABLE OF CONTENTS

Chapter I: General Introduction

1.1 Introduction.....1

Chapter II: Literature Review

2.1 Transforming Growth Factor-β

2.1.1 Introduction.....4

2.1.2 TGF-β Promoter.....5

2.1.3 TGF-β Structure.....6

2.1.4 Activation of TGF-β.....7

2.1.5 Effects on Target Cells.....9

2.1.6 Effects on Extracellular Matrix.....10

2.1.7 TGF-β Null Mice.....11

2.1.8 TGF-β and Other Cytokines.....12

2.1.9 Regulation of TGF-β.....13

2.1.10 Receptor for TGF-β.....15

2.2.11 Modulation of the Immune System by TGF-β.....16

2.2 Extracellular Matrix

2.2.1 Roles of TGF-β in Extracellular Matrix Formation.....19

2.2.2 TGF-β and Wound Healing.....20

2.2.3 Scarless Wound Healing.....21

2.2.4 Estrogen and TGF-β.....23

2.2.5 TGF-β and Other Cytokines in Wound Healing.....24

2.3 TGF-β Transgenic Models

2.3.1 Transgenic Animals.....27

2.3.2 Developing Transgenic Animals.....27

2.3.3 Keratin 14 Promoter.....28

2.3.4 Basement Membrane.....29
2.3.5 TGF-β and Transgenic Animals.....30

2.4 TGF-β and Interferons
2.4.1 Introduction to Interferons.....34
2.4.2 Functions of IFNs.....34
2.4.3 Interactions Bewteen TGF-β and IFN-γ.....35

2.5 Effects of TGF-β on Th_1 and Th_2 Cells
2.5.1 Introduction to Th_1 and Th_2.....39
2.5.2 Differentiation of Th_1 and Th_2.....40
2.5.3 Th_1Stimulation by TGF-β.....41
2.5.4 Th_1 Apoptosis Delayed by TGF-β.....42

2.6 Summary and Hypothesis.....43

Chapter III: Material and Methods
3.1 Transgene Construction and the Production of Transgenic Animals.....47
3.2 DNA Isolation.....48
3.3 Southern Blotting.....48
3.4 Extraction of RNA from Skin and Wounds.....49
3.5 Reverse Transcription.....50
3.6 Polymerase Chain Reaction.....51
3.7 Quantitative Enzyme Immunoassay for TGF-β1.....51
3.8 Wound Healing Model.....53
3.9 PAI/L Assay for *In Vivo* TGF-β Determination.....54
3.10 Northern Blotting.....54
3.11 Immunohistochemistry.....55
3.12 Hydroxyproline Analysis.....56

Chapter IV: Results

4.1 *In Vitro* Testing of the pG3Z:K14-TGF-β1 Construct.....57

4.2 Screening for Transgene.....58

4.3 Breeding a Transgenic Mouse Homozygous for the K14-TGF-β1 Construct.....58

4.4 Reverse-Transcription Polymerase Chain Reaction for Human TGF-β1 mRNA.....59

4.5 Phenotypic Changes.....60

4.6 Serum TGF-β1.....60

4.7 Wound Healing.....61

4.8 TGF-β1 and α1-Procollagen mRNA Expression by Northern Analysis...62

4.9 Hydroxyproline Content of Skin and Wound Samples.....64

4.10 Histology and Immunohistochemistry.....65

4.11 TGF-β1 in the Serum.....66

4.12 PAI/L Assay for *In Vivo* TGF-β1.....67

Chapter V: Discussion.....68

Chapter VI: Conclusions and Future Applications

6.1 Conclusions.....77

6.2 Future Applications.....78

Literature Cited.....81

LIST OF FIGURES AND APPENDICES

Figure 1. Plasmid construct used to make the transgene.....104

Figure 2. Transgenic pedigree.....105

Figure 3.

A.Generation of homozygous animals.....106

B.Southern blotting results of progeny from testing of potential homozygous animals.....106

Figure 4. Reverse-transcription polymerase chain reaction (RT-PCR).....107

Figure 5. Phenotypic changes.....108

Figure 6.

A. Masson's trichrome staining of skin cross sections.....109

B. Hydroxyproline levels in skin (day 0).....109

Figure 7. Serum TGF-β1 protein levels (day 0).....110

Figure 8. Wound healing (Re-epithelialization photos).....111

Figure 9. Wound closure (Graph).....112

Figure 10. Human TGF-β1 northern analysis.....113

Figure 11. Mouse TGF-β1 northern analysis.....114

Figure 12. Proα1 (type I) collagen northern analysis.....115

Figure 13. Type III collagen northern analysis.....116

Figure 14. Hydroxyproline content post-wounding.....117

Figure 15. Histology (day 5).....118

Figure 16. Histology (day 7).....119

Figure 17. Histology (day 9).....120

Figure 18. Serum TGF-β1 levels post-wounding.....121

Figure 19. PAI/L assay for *in vivo* TGF-β1.....122

Appendix I. Homozygous breeding numbers.....124

Appendix II. Human TGF-β1 PCR product sequence.....125

Appendix III. Mouse TGF-β1 PCR product sequence.....126

Appendix IV. Wound re-epithelialization (raw data).....127

Appendix V. Scanned northern analysis (graphs).....128

Appendix VI. Biopsy wet weights.....129

Appendix VII Biopsy dry weights.....130

Appendix VIII Percentage of water.....131

Appendix IX Raw data (northern scans).....132

LIST OF ABBREVIATIONS

ANOVA	analysis of variance
bFGF	basic fibroblast growth factor
bp	base pair
cDNA	complementary deoxyribonucleic acid
ECM	extracellular matrix
EGF	epidermal growth factor
GITC	guanidium isothiocyanate
HSc	hypertrophic scar
IFN-γ	interferon-gamma
IGF-1	insulin-like growth factor
IL	interleukin
LAP	latency associated peptide
LTBP	large latency binding protein
M-6-P	mannose 6-phosphate
MRNA	messenger ribonucleic acid
NO	nitric oxide
PAI/L	plasminogen activator inhibitor-1/luciferase
PDGF	platelet-derived growth factor
RT-PCR	reverse transcription-polymerase chain reaction
TGF-β	transforming growth factor-beta
TIMP	tissue inhibitor of metalloproteinases
TNF	tumor necrosis factor

CHAPTER I

GENERAL INTRODUCTION

1.1 INTRODUCTION

Fibrosis is characterized as excessive deposition of connective tissue during the process of wound healing. This may occur in organs such as the liver and lungs and when it occurs in the skin, it may lead to a condition called hypertrophic scarring which results from deep dermal injury followed by a prolonged inflammatory and fibroplastic phase of wound healing. A hypertrophic scar may burden the patient with decreased mechanical flexibility and movement after injury.

Both hypertrophic scars and keloids are characterized by excessive production of collagen in the dermis and subcutaneous tissues arising from injuries (Tuan et al, 1998). Complications associated with these scars include disfigurement, contractures, pruritis and pain (Tuan et al, 1998). Major differences between these two wounds lie in the fact that hypertrophic scars remain within the original wound boundaries and regress over time, while keloids will grow beyond the original wound size, eventually overtaking normal tissue, and do not regress (Tuan et al, 1998).

In vitro testing of fibroblasts isolated from hypertrophic scars indicate that the cytokine transforming growth factor-beta (TGF-β) has an important role in the production of hypertrophic scars. The study of hypertrophic fibroblasts in collagen lattices reveal that TGF-β1 could enhance contraction in these gels, but no differences between normal and hypertrophic fibroblasts were seen when these cells were treated with EGF, PDGF or bFGF (Yang et al, 1997). Fibrin clot contraction studies *in vitro* also demonstrated that hypertrophic scars exhibited stronger contractions over normal and

keloid fibroblasts (Younai et al, 1996). Interestingly, this effect could be neutralized with the addition of anti-TGF-β antibodies. TGF-β is able to induce the expression of smooth muscle actin (α-SM) which is found in abundance in hypertrophic fibroblasts (Desmouliere et al, 1993; Tredget et al, 1997). α-SM is involved in cellular adhesion and locomotion. In patients with substantial amounts of hypertrophic scarring, it has been shown that serum TGF-β is elevated (Tredget et al, 1997).

TGF-β is a cytokine with recorded effects on many cell types ranging from epithelial to immune cells. Its role is crucial in processes such as wound healing and especially in extracellular matrix formation. It can be found within many tissues but especially platelet granules and bone (Roberts et al, 1993). The effects of TGF-β in upregulating extracellular matrix synthesis (collagen, fibronectin) and indirectly inhibiting proteinase activity (increased plasminogen activator inhibitor-I, tissue inhibitor of metalloproteinases; decreased collagenase, plasminogen activator) suggests it may be the key cytokine in hypertrophic scar formation. Transforming growth factor-beta has the potential to be responsible for many fibrotic conditions. In addition to its roles in matrix metabolism, it also has profound effects on the immune system. With the help of transgenic animal models, studies may be performed on the role of TGF-β in extracellular matrix formation and its interaction with the immune system and other interferons.

It would be of great benefit to physicians to be able to control the process of wound healing in order to prevent the formation of hypertrophic scars. One area of interest is the study of the mechanisms involved in extracellular matrix formation. Understanding the cytokines that promote collagen deposition may eventually allow the prevention of fibrosis by administration of the proper antibodies to these cytokines. Transforming

growth factor-beta 1 stimulates extracellular matrix synthesis at the same time inhibiting matrix degradation (Ebner et al, 1993; Lindsey et al, 1991). Our hypothesis is that excessive TGF-β production is a major factor in the pathogenesis of hypertrophic scarring. The study of a genetically manipulated animal, which produces excessive TGF-β in the skin, would be useful to evaluate its role in the epidermis and may help to further understand the development of fibrosis.

This thesis can be summarized into two major experimental areas. First, we decribe the generation of a novel TGF-β1 transgenic mouse, using the keratin 14 promoter to target a latent TGF-β1 transgene product. Characterization of this mouse was performed by observing phenotypic and microstructural changes, detecting specific mRNA expression in different organs, measuring hydroxyproline in skin biopsies and serum TGF-β1. The founders were heterozygous for the transgene but we were successful in breeding a line of homozygous animals.

The second major part of this thesis involved studying the effects that this transgene would have on wound healing in these animals. Parameters studied included re-epithelialization of the wounds, Northern analysis, hydroxyproline content, immunohistochemstry for TGF-β1 protein, serum TGF-β1 protein levels and *in vivo* measurement of TGF-β1 by the PAI/L biological assay.

CHAPTER TWO

LITERATURE REVIEW

2.1 TRANSFORMING GROWTH FACTOR-β

2.1.1 Introduction

Transforming growth factor-β was named from its initial identification *in vitro* from the conditioned medium of transformed cells (De Larco et al, 1978; Roberts et al, 1980) but is now known to be in most tissues (Assoian et al, 1983; Roberts et al, 1993) and especially in platelets (Assoian et al, 1983; Childs et al, 1982; Roberts et al, 1993). In activated lymphocytes, levels of TGF-β rise 10-30 fold over those in non-activated cells (Kehrl et al, 1986). TGF-β was originally characterized based on its relationship with the cytokine epidermal growth factor (EGF) (Sporn et al, 1983). TGF-β requires EGF to induce colony formation of untransformed indicator cells in soft agar. TGF-α, on the other hand, competes with EGF for receptor binding, but does not require EGF for colony formation (Sporn et al, 1983). Today, there have been numerous other molecules discovered to be similar to TGF-β and belong to the same superfamily of cytokines. Each molecule has been characterized by its homology to TGF-β1 (Chambaz et al, 1996). TGF-β2 and β3 possess 75-80% sequence identity; whereas, molecules that have 30-40% sequence identity include activin and inhibin (a combination of two different A and B monomers); bone morphogenetic proteins (BMPs 1-6); growth and differentiation factors (GDF 1-3); and Mullerian inhibiting hormone (MIH, MIS). On the far end of the scale, glial-derived neurotrophic factor (GDNF) has only 15-30% sequence identity to TGF-β1.

TGF-β sequence is highly conserved between the five subclasses and within species (Ghahary et al, 1996) but only TGF-β1, β2, and β3, have been found in mammals (Van Obberghen-Schilling et al, 1988).

2.1.2 TGF-β Promoter

TGF-β is not only interesting because of the effects the protein has on mature and undifferentiated cells, but also the gene possesses properties that provide invaluable insight to its function. There are two transcriptional origins 271 nucleotides apart. The gene also contains two promoter regions: one is 1400 basepairs 5' upstream from the 5'-most transcriptional start site and the other is located between the two transcriptional start sites. TGF-β is also capable of autoinduction of its own signal (McCartney-Francis et al, 1990). Autoregulation appears to be from a 130 base-pair fragment that is located between -453bp and -323bp upstream from the 5'-most start site and a fragment between the two promoters (Kim et al, 1990).

The human TGF-β1 gene consists of two TATA-box-like structures (at positions -71bp and -356bp upstream), 9 hexanucleotide repeat sequences (either CCGCCC or GGGCGG), 2 major transcriptional start sites (+1bp and +271bp) and negative regulatory elements (Kim et al, 1989). Deletional analysis reveals the negative regulatory regions located between -1362bp to -1132bp and also at -731bp to -433bp suppresses transcription. There is also an enhancer-like element situated between -1132bp and -731bp which has the opposite effect. The second promoter can be found downstream from the start site (+1bp to +271bp) and is also required for human TGF-β expression. Hexanucleotide repeat sequences at positions -32bp, -43bp, -78bp, -108bp, -120bp

-176bp, -218bp, -235bp, and -313bp are similar to those found in viral and cellular housekeeping genes.

2.1.3 TGF-β Structure

TGF-β is synthesized as a dimeric proprotein (Ghahary et al, 1996; Gleizes et al, 1997) consisting of the active TGF-β (25kDa) and an N-terminal precursor peptide or latency associated peptide (LAP)(74kDa) (Chambaz et al, 1996; Gleizes et al, 1997; Rossi et al, 1988). Mature TGF-β is the C-terminal position of the proprotein (amino acids 280-392) whereas the LAP is the N-terminal position and is a glycosylated dimer that binds mature TGF-β by noncovalent interactions. Interchain disulfide bonds at cysteines 223 and 225 (Chambaz et al, 1996; Gleizes et al, 1997) of the LAP are necessary for the latency to TGF-β. Site mutations substituting serines for cysteines at positions 223 and 225 will lead to constitutively active TGF-β (Brunner et al, 1989; Cui et al, 1995; Kopp et al, 1996; Samuel et al, 1993; Sanderson et al, 1995; Sellheyer et al 1993). There are also mannose 6-phosphate (M-6-P) residues at asparagines 82, 136 and 179 (Gleizes et al, 1997). This is important because M-6-P is normally used as a signal for lysosomal targeting through the M-6-P receptors and may play a role in TGF-β activation. The biologically active form requires cleavage of the mature peptide from LAP to produce a 25kDa dimer (the only active form of TGF-β) stabilized by disulfide bonds holding the 112 amino acid subunits together (Bronzert et al, 1990; Ghahary et al, 1996; Sporn et al, 1986; Van Obberghen-Schilling et al, 1988). *In vivo*, TGF-β can also be found with a 120-240 kDa glycoprotein designated latent TGF-β binding protein

(LTBP) which associates with the LAP through disulfide bonds (Gleizes et al, 1997). Due to its latency, increased levels of TGF-β do not always indicate increased levels of active TGF-β (Theodoresu et al, 1991). The function of TGF-β is mediated through receptors and its interaction with other growth factors; but the exact pathway of its intracellular signaling pathway is still unknown. The receptors for TGF-β are associated with transmembrane serine/threonine kinases that induce signalling through heterologous phosphorylation by the RI and RII receptors (Chambaz et al, 1996; Chen and Weinberg, 1995; Hall et al, 1996; Wrana et al, 1994). Receptor phosphorylation events are believed to also phosphorylate, and thus activate Smad2 and Smad3 (Zhou et al, 1999). Smads are genes that are associated with the intracellular signalling of TGF-β. The activated Smads in combination with Smad4 translocate to the nucleus where they recruit other transcription factors and bind to specific Smad binding sites within their promoter regions (Zhou et al, 1999).

2.1.4 Activation of TGF-β

Activation *in vitro* can be caused by heat (80°C for 10 minutes), pH changes (extremely acidic or basic), and detergents (Border et al, 1992; Gleizes et al, 1997). The activation of TGF-β *in vivo* is not yet fully understood. The cleavage of the LAP from the mature TGF-β to release the active dimer is usually a necessary step and may involve proteinases (such as plasmin and cathepsin D) which will cleave the LAP in the N-terminal region (Lyons et al, 1988; 1990). The site of TGF-β activation is most probably at the cell surface where the latent TGF-β is localized by insulin-like growth factor

II/mannose 6-phosphate receptor and plasmin is protected from inhibitors (Gleizes et al, 1997). Studies show that when a large excess of M-6-P is added to cultures, an inhibition of TGF-β activation occurs (Dennis et al, 1991). The LTBP is similar in structure to fibrillin (Gleizes et al, 1997; Taipale et al, 1996) (a protein found in elastic fibers) and may help regulate TGF-β by masking the M-6-P moieties of latent TGF-β. LTBP is also seen to help direct latent TGF-β to the extracellular matrix (ECM) as observed in cells producing the latent complex (Taipale et al, 1994; 1996). When LTBP is associated with the ECM, it is bound by covalent crosslinking through the action of transglutaminase and can not be recovered by denaturing agents (Nunes et al, 1997). Only by the enzymatic cleavage of the LTBP can it be separated from the ECM (Nunes et al, 1997; Taipale et al, 1994; 1995).

The most logical steps of activation involve 1) the targeting of the large latent complex (consisting of LTBP-LAP-TGF-β) to the ECM and covalently linking to it by the action of transglutaminase; 2) proteolytic cleavage of the LTBP releasing LAP-TGF-β and exposing M-6-P residues; 3) cell surface IGF-II/M-6-P receptors binding the LAP and making the latent complex more susceptible to the action of plasmin; 4) cleavage of the LAP from TGF-β by plasmin releasing the active dimer. Other enzymes worth noting are cathepsin D, which can also cleave the LAP. LTBP can be released from the ECM by mast cell chymase, leukocyte elastase and thrombin (Gleizes et al, 1997). Activation is not exclusive to the IGF-II/M-6-P receptor pathway as activation independent of this receptor has been noted in endothelial cells (Kojima and Rifkin, 1993) and human osteoblast-like cells (Oursler et al, 1993) when treated with retinoic acid. There are also some data to suggest a role of thrombospondin-1 (TSP-1), an adhesive molecule that

binds cell surfaces and ECM and is also present in the α granules of platelets (Gleizes et al, 1997). Addition of TSP-1 to endothelial cell cultures yields active TGF-β (Shultz-Cherry and Murphy-Ullrich, 1993); probably through a non-enzymatic process that involves the interaction of the properdin-like (type I) domains of TSP-1 with latent TGF-β (Shultz-Cherry and Murphy-Ullrich, 1994). These domains consist of three repeats of approximately 60 residues and all repeats contain the consensus sequence WSXW found in the TSP family and also in members of cytokine receptors and TGF-β superfamilies (Shultz-Cherry and Murphy-Ullrich, 1994).

2.1.5 Effects on Target Cells

The mRNA coding for TGF-β is constitutive in both normal and experimentally transformed cells (Glick et al, 1993) and its proliferative effects are very much dependent on the target cells. Inhibition of growth by TGF-β can be seen in epithelial cells, endothelial cells, lymphocytes of both B and T lines, hepatocytes, embryo fibroblasts and in (experimental studies) normal and cancerous mammary epithelial cells (Anzano et al, 1986; Roberts et al, 1993; Sporn et al, 1986). Differentiation is also inhibited by TGF-β in preadipocytes, myoblasts and lymphocytes. TGF-β can actually stimulate growth and activation in mesenchymal cells (Kim et al, 1989) and chondroblasts (Li et al, 1993) and promotes a more mature phenotype in human mammary epithelial cells (Sporn et al, 1986). TGF-β has many cellular targets and it also induces a broad response from different cell types. Studies involving keratinocytes have shown that TGF-β appears to inhibit DNA synthesis or induce apoptosis in the suprabasal layer of the epidermis.

It is generally known as a negative regulator for cell growth (including bone marrow cells and fetal liver cells *in vitro*) (Gleizes et al, 1997; Sachs et al, 1996) but these effects may be dependent upon the concentration of TGF-β present and the resting state of the cell. TGF-β stimulates resting cell types and may inhibit active forms of the same cells (Wahl, 1994). The effect of TGF-β on a given cell type are highly dependent on the differentiation state of the target cell and the other growth factors present (Chambaz et al, 1996).

2.1.6 Effects on Extracellular Matrix

The function of TGF-β was originally defined by its ability to transform cells that are anchorage-dependent to an anchorage independent growth (Hebda, 1988). TGF-β1 and β2 are more widely known for their ability to promote matrix production by increasing the transcription of matrix genes (fibronectin, collagen type I, biglycan), increasing the stability of extracellular matrix RNA and decreasing transcription of matrix-degrading enzymes to reduce ECM proteolysis (Gleizes et al, 1997; Zhang et al, 1995).

Studies *in vitro* of the effects of TGF-β on different cultured cells have helped define its activities. TGF-β1 was shown to increase elastin production by increasing mRNA stability in fibroblasts and smooth muscle cells (Zhang et al, 1995). It inhibits proliferation of epithelial cells while promoting ECM production *in vitro*, and granulation tissue formation *in vivo* (Davidson et al, 1993). In fibroblasts, TGF-β has been shown to increase the production of collagen and fibronectin (Hebda, 1988; Igotz and Massague, 1986; Roberts et al, 1986; Varga and Jimenez, 1986).

Fibroblast growth factor-2 and retinoic acid added to endothelial cell cultures stimulate TGF-β production (Flaumenhaft et al, 1992; Kojima et al, 1993). *In vivo*, TGF-β is released from the α granules of platelets during blood clotting (Sporn et al, 1986). Excess TGF-β causes unresolved inflammation and fibrosis at sites of injection locally (Sporn et al, 1986; Wahl, 1994), but systemically it will inhibit the expression of E-selectin in endothelial cells to prevent leukocytes from adhering and targeting the site of inflammation (Gamble et al, 1993). It inhibits the production of nitric oxide (NO) and downregulates NO synthetase in macrophages (Wahl, 1994). TGF-β also has a mechanism of autoinduction; the presence of TGF-β will increase its own production and secretion (McCartney-Francis et al, 1990; Letterio et al, 1994).

2.1.7 TGF-β Null Mice

To further understand the role of TGF-β *in vivo*, mice were generated with deletions of areas in the TGF-β gene. TGF-β null mice gave birth to normal pups, but at 2-3 weeks of age, leukocyte infiltration into the organs of newborn animals led to death (Kulkarni et al, 1993). Other groups have reported that animals homozygous for the TGF-β null genotype died early with cardiac abnormalities (Letterio et al, 1994); yet heterozygous animals bearing homozygous offspring can delay the lethal phenotype by the transfer of TGF-β across the placenta from the maternal circulation (Letterio et al, 1994). Due to its effects on many different cell types, it was expected that TGF-β knock-out mice would show signs of severe systemic inflammation affecting different organ systems (which was observed within 1 week of life and resulted in wasting and death at 4 weeks of age) (Kulkarni et al, 1993; Letterio and Roberts, 1996). To show that the lethal phenotype

(Kulkarni et al, 1993) was not due to any secondary complication arising from the nonlocalized inflammation, knock out mice were bred to SCID mice (Diebold et al, 1995) (to avoid the inflammatory process) and were able to survive. Knock-out mice with deletions in the plasminogen activator genes do not express the same phenotype as the TGF-β knock out mice indicating that other mechanisms, not only plasmin, are involved in TGF-β activation (Gleizes et al, 1997). Knock-out mice have shown that although TGF-β can promote the inflammatory cytokine production (recruitment phase) it is its ability to control these processes (resolution phase) that is more important (Letterio and Roberts, 1996).

2.1.8 TGF-β and Other Cytokines

Interactions between TGF-β and other cytokines are very important in understanding its function *in vivo*. Basic fibroblast growth factor (b-FGF), generally associated with vascular endothelial cells, can stimulate expression of plasminogen activator and neutral metalloproteinases (Davidson et al, 1993). The addition of b-FGF can inhibit TGF-β1 and TGF-β2 stimulated elastin and collagen production in normal skin fibroblasts and smooth muscle cells (Brettell and McGowan, 1994; Davidson et al, 1993; Zhang et al, 1995). In addition to b-FGF, TGF-α was another potent antagonist to TGF-β1, but there is evidence of a biphasic effect of cytokine concentration (Davidson et al, 1993). Tumor necrosis factor-alpha (TNF-α) is also antagonistic to TGF-β by reducing the activity of the elastin promoter (Kahari et al, 1992). Insulin-like growth factor-1 (IGF-1) is a direct growth mitogen and also enhances the synthetic pathways (protein synthesis and ECM

production) of fibroblasts and smooth muscle cells, but its addition to cell cultures only slightly enhanced elastin production and had no effect in collagen (Davidson et al, 1993). Epidermal growth factor (EGF) is also known to be a potent mitogenic activator (Davidson et al, 1993) and has been shown to improve the initiation of cell outgrowth from porcine skin explant cultures over the use of TGF-β alone (Hebda, 1988). But other studies *in vitro* show EGF to have antagonistic effects to TGF-β's induction of collagen in cultured fibroblasts by a separate mechanism of EGF's ability to induce collagenase (Roberts et al, 1986).

2.1.9 Regulation of TGF-β

The control of TGF-β *in vivo* is as important as its activation. We have already discussed some of the different cytokine interactions on the activity of TGF-β and now look at other ways of controlling TGF-β.

Active TGF-β was seen to be degraded mostly in the liver, kidneys, lungs and spleen (Wakefield et al, 1990). The half-life of TGF-β in plasma in rats is only 2-3 minutes (Gleizes et al, 1997). Latent TGF-β was kept in the circulation and 90% was not degraded after 90 minutes. With reduced degradation in the plasma, this would allow TGF-β to travel in the circulation and work in more distal sites from its production, thus promoting a more endocrine over autocrine/paracrine activity. This stability was heavily dependent on the sialylation of the LAP because complexes made with insufficient sialylation had an even shorter half-life than active TGF-β (1.2 ± 0.3 min) (Wakefield et al, 1990). The normal function of the asialoglycoprotein receptors in the liver is to collect aged desialylated plasma proteins (Ashwell and Hartford, 1982) and may be the

mechanism of TGF-β clearance. It was also noted that the overall distribution of TGF-β is very similar to α-2-macroglobulin (α2M) (Feldman et al, 1985; Okubo et al, 1984) and supports the idea that this is another mechanism by which the liver can clear circulating TGF-β when it is complexed with α2M (O'Connor-McCourt and Wakefield, 1987).

TGF-β activation *in vitro* in co-cultures of endothelial cells and smooth muscle cells emphasize the role of plasmin since TGF-β activation can be blocked by plasmin inhibitors (Sato and Rifkin, 1989). This mechanism is self-regulating as TGF-β upregulates plasminogen activator inhibitor-1 (PAI-1), which decreases latent TGF-β activation by inhibiting urokinase type plasminogen activator (u-PA) (Gleizes et al, 1997).

Decorin is a natural proteoglycan that regulates TGF-β by binding to it (Border et al, 1992; Yamaguchi et al, 1990). TGF-β has been implicated in cases of nephrosclerosis (Border et al, 1992), experimental glomerulonephritis (Okuda et al, 1990), proliferative vitreoretinopathy and systemic sclerosis in the skin (Border et al, 1992; Connor et al, 1989; Kulozik et al, 1990; Okuda et al, 1990). The administration of decorin has been shown to inhibit the increased matrix production and thereby prevent the disease manifestations (Border et al, 1992). Decorin inhibits the action of TGF-β1, -2, -3 (Border et al, 1992).

There are other ways to inhibit TGF-β activity. Apo(a), the protein found in lipoprotein(a), is structurally similar to plasminogen and therefore can decrease the formation of TGF-β (Gleizes et al, 1997). Transgenic animals expressing apo(a) have lower TGF-β and plasmin levels (Grainger et al, 1994; Lawn et al, 1996). Studies have shown that TGF-β activation can be inhibited by excessive LTBP-1, antibodies to LTBP

(Flaumenhaft et al, 1993), or inhibitors of transglutaminase (Kojima et al, 1993). Antibodies to the N-terminal region of the LTBP prevents its association with transglutaminase which in turn prevents incorporation of TGF-β into the ECM (Gleizes et al, 1997).

2.1.10 Receptors for TGF-β

There are three types of cell surface receptor for TGF-β: Type I or RI (50-60kDa), Type II or RII (70-85kDa), and Type III or RIII (200-400kDa) (Chambaz et al, 1996; Hall et al, 1996). RI and RII belong to the family of transmembrane serine /threonine kinases. The function of both receptors are required for signalling (Bloom et al, 1996) because RI only binds TGF-β when RII is co-expressed (Chambaz et al, 1996). RI and RII form heterooligomeric complexes to bind TGF-β (Wrana et al, 1992). Although there are several Type I receptors, only one functional Type II receptor has been identified (Lin et al, 1992). RI and RII share the same overall molecular structure with similar extracellular cysteine-rich and intracellular serine/threonine specific protein-kinase sequences (Ebner et al, 1993). RII is the primary TGF-β receptor that activates RI serine-threonine kinase after binding to ligand by heterologous phosphorylation (Chambaz et al, 1996; Chen and Weinberg, 1995; Hall et al, 1996; Wrana et al, 1994). RII can also form homodimers and its cytoplasmic domain may be autophosphorylated independently of ligand (Chen and Weinberg, 1995; Wrana et al, 1994). Signaling requires a functioning RI kinase domain, but RII is more important in mediating TGF-β function (Bloom et al, 1996; Hall et al, 1996).

RIII or betaglycan is a transmembrane proteoglycan and does not directly participate in TGF-β signalling (Chambaz et al, 1996; Hall et al, 1996). Its role involves presenting TGF-β to RII to increase the affinity of the RII receptors for TGF-β (Lopez-Castilla et al, 1993).

Although not yet fully understood, intracellular signalling activities involve Smad proteins (Zhou et al, 1999). Specifically Smad2 and Smad3 become activated by phosphorylation after receptor-ligand binding. As the activated Smads translocate to the nucleus, they recruit Smad 4 and other transcription factors. In the nucleus, this complex of Smads and transcription factors associate with binding sites in the promoter region and promote gene expression (Zhou et al, 1999).

2.1.11 Modulation of the Immune System by TGF-β

Based upon its anti-proliferative functions on most cells, TGF-β has profound effects on the immune system. It generally suppresses the differentiation and the proliferation of B and T cells and antagonizes the action of proinflammatory cytokines such as interleukin-1β, -2, -6, tumor necrosis factor-α, and interferon-γ (Espevik et al, 1990; Roberts et al, 1993). Other anti-inflammatory effects include inhibiting the production of superoxide and nitric oxide in macrophages (Roberts et al, 1993; Vodovotz et al, 1993). TGF-β can inhibit expression of interleukin -1 and -2 receptors on cells (Roberts et al, 1993). TGF-β is also seen to have proinflammatory as well as anti-inflammatory effects. It works as a chemoattractant and promotes the diapedesis of cells such as monocytes, T-cells and neutrophils (Wahl et al, 1987). TGF-β has also been reported to prevent adhesion to endothelial cells by neutrophils and T-cells (Roberts et al, 1993; Wahl et al,

1993). Lymphocytes can be concentrated at specific sites and traverse blood vessels through integrin interactions which are increased by TGF-β. Integrins are important for cells to bind and respond to the extracellular matrix. They are composed of 2 noncovalently associated transmembrane glycoprotein subunits (alpha and beta), and both subunits contribute to the binding of the matrix proteins (Hynes, 1987).

Another method to study the importance of TGF-β in the immune system is to examine the abnormalities that arise from changes in TGF-β levels. Loss of TGF-β expression gives rise to many disease states attributed to the dysregulation of the immune system. In one study with papillomas and carcinomas (Glick et al, 1993), loss of TGF-β1 or TGF-β2 gave rise to either basal cell proliferation or suprabasal hyperproliferation respectively. The cells studied had significant decreases in TGF-β protein levels, but normal to high levels of mRNA. A post-transcriptional mechanism must therefore be responsible for the changes in TGF-β levels.

Studies on mice bearing tumors in spleen cells helped to developed a theory about the role of TGF-β in immunosuppression (Li et al, 1993). It was proposed that the defect seen in $CD4^+$ T-helper (Th) cells in their response to normal antigens were due to levels of active TGF-β. The inactivity of the $CD4^+$ Th cells arose from recognition of the processed antigens which were associated with self Ia molecules. Recombinant TGF-β inhibited secretion of lymphokines (IL-2, macrophage-activating factor/interferon-gamma) in culture depending on the stage of the tumor. Early stages produced low levels of lymphokines and late stage tumors produced none. In contrast, the plasma levels of TGF-β rose at later stages of tumor development. It appeared that TGF-β had a greater suppressive effect upon the $CD4^+$ Th cells in late stage tumor-bearing mice. Both the Th_1

and Th_2 subclasses were compromised at the later stages of tumor development suggesting that suppressive mechanisms of TGF-β upon immune cells are critical to tumor formation.

Autoimmunity is another important area for the study of TGF-β. Animal models of multiple sclerosis (experimental autoimmune encephalomyelitis, EAE) (Racke et al, 1992) and rheumatoid arthritis (Kuruvilla et al, 1991) have been studied. Studies on EAE have revealed that TGF-β can delay the onset of the disease, decrease the severity of relapses that occur during the primary disease and remission, and play a critical role in the recovery and remission of EAE. These findings were supported by experiments using specific anti-TGF-β antibodies (Miller et al, 1992; Racke et al, 1992) that increased disease severity. TGF-β is released in its active form by $CD8^+$ cells as a response to TGF-β from activated T cells (Karpus et al, 1991).

2.2 EXTRACELLULAR MATRIX

2.2.1 Roles of TGF-β in Extracellular Matrix Formation

One of the most important applications of TGF-β is in wound healing; a process of tissue repair that involves stages of inflammation, cell proliferation, angiogenesis and the synthesis and re-organization of extracellular matrix (ECM) (Amento and Beck, 1991). TGF-β affects transcription for matrix proteins and their enzyme inhibitor genes which results in the accumulation of ECM. Autoinduction mechanisms are important to amplify the effects of TGF-β in ECM deposition.

The matrix proteins whose synthesis is stimulated by TGF-β include fibronectin, certain proteoglycans and collagen (Van Obberghen-Schilling et al, 1988; Varedi et al, 1995). TGF-β will increase the steady-state levels of RNA for these proteins, thus increasing their translation (Van Obberghen-Schilling et al, 1988). The proteinase inhibitors plasminogen activator inhibitor-1 (PAI-1) and tissue inhibitor of metalloproteinases (TIMPs) are increased (Van Obberghen-Schilling et al, 1988; Varedi et al, 1995); whereas the matrix proteinases themselves are decreased (e.g. collagenase, plasminogen activator) (Flaumenhaft et al, 1992; Varedi et al, 1995). These proteinase inhibitors act to confine the action of proteinases to specific areas (Birkedal-Hansen et al, 1992). TGF-β1 binds to the following matrix components that may initiate activation or inhibit its activity: thrombospondin, fibronectin, decorin and biglycan (proteoglycans) (Benn et al, 1996).

Fibroblasts are the primary focus for TGF-β in matrix formation. Increased transcription of fibroblast ECM proteins (type I, III and V collagen, fibronectin) is

through a Nuclear Factor I binding site used to upregulate the steady-state level of the mRNA (Rossi et al, 1988). In addition to its effects on transcription, TGF-β is a potent chemotactic factor and promotes proliferation of immature fibroblasts at the site of injury (Rossi et al, 1988).

The most concentrated source of TGF-β is the granules of platelets (which release their contents at the time of injury) but it is most abundantly found in bone. It promotes growth of osteoblasts *in vitro* and enhances production of collagen by these cells (Robey et al, 1987). It may also be involved in osteoporosis since the low levels of TGF-β correlate with the decreased vitamin D concentrations in rat models (Finkelman et al, 1991).

2.2.2 TGF-β and Wound Healing

The normal time course of wound healing involves cellular migration and inflammation at 0-7 days post-wounding; proliferation of fibroblasts, collagen synthesis between 2-4 weeks and remodelling (collagen cross-linking and active collagen turnover) can last from 1 month to 1 year. Normal collagen deposition has been described as a "basket-weave" organization (Ashcroft et al, 1997). Fibers are generally laid parallel to the epidermis. Due to its abundance in platelet α granules, it was first speculated that TGF-β plays a role in wound healing. TGF-β can stimulate fibrosis when injected in rats (Sporn et al, 1983) and newborn mice (Roberts et al 1986). When administered to incisional wounds, it has been reported to increase the matrix components and thus produce increased tensile strength (Roberts et al, 1993; Benn et al, 1996). This was observed by injections of TGF-β directly into the wounds (Roberts et al, 1986) or even

through cytomegalovirus (CMV) transfections of granulation tissue with latent TGF-β1 cDNA (Benn et al, 1996). In the case of CMV transfections, there was increased collagen concentration without changes in cellularity in the incisional wounds (Benn et al, 1996). Generally, TGF-β produces increased infiltration of mononuclear cells and fibroblasts, collagen synthesis and tensile strength during wound healing (Benn et al, 1996; Mustoe et al, 1987; Roberts et al,1993).

2.2.3 Scarless Wound Healing

Many fetal wounds heal without scars (Coleman et al, 1998). This is believed to be from the fetal fibroblasts depositing collagen in a reticular pattern (Lorenz et al, 1995), as opposed to a more random deposition of fibers (as seen in scars) laid down by adult fibroblasts. TGF-β also promotes wound contraction and it was hypothesized that due to the lack of TGF-β in fetal wounds (Whitby et al, 1991), the wounds healed without contraction and this is a major mechanism for scarless healing (Coleman et al, 1998). When testing this hypothesis, the addition of exogenous TGF-β into fetal wounds induced contraction and scarring (Lin et al, 1995; Piscatelli et al, 1994) and the addition of TGF-β1 antibodies resulted in scarless healing in rats (Sullivan et al, 1995).

When human fetal and adult skin was grafted onto athymic mice, only the adult skin produced scarring (characterized by random collagen formation) after incisional wounding (Lin et al, 1995). Immunohistochemistry has shown decreased TGF-β1 staining in the fetal wounds (Shah et al, 1992). Characteristics of the fetal wounds included undetectable levels of TGF-β mRNA by *in situ* hybridization, the lack of macrophages and neutrophils from human or murine sources and the predominant cell

type was fibroblasts (Lin et al, 1995). Although TGF-β mRNA is constitutively expressed in the basal layer of the epidermis and hair follicles, there was an absence of both latent and active TGF-β1 protein in the fetal skin wounds (Sullivan et al, 1995). The addition of exogenous TGF-β1 was through a cellulose disk that slowly released the cytokine over 14 days placed between the mouse fascia and the human dermis. Fetal wounds treated with excess TGF-β produced an induction of TGF-β mRNA in the human fibroblasts in the graft, murine macrophage and neutrophil infiltration to the injury site and healed with the same disorganized collagen deposition, characteristic of scar formation, as the adult skin grafts (Lin et al, 1995). These results correlate with other studies that showed low subcutaneous doses of TGF-β1 induce fibrosis and are chemotactic for fibroblasts, macrophages and granulocytes in adult wound models (Roberts et al, 1986). Other animal models showed similar results. In rabbits, polyvinyl sponges impregnated with TGF-β produced fibrosis when placed under the skin (Krummel et al, 1988). In an adult pig excision model, TGF-β1 injections were shown to increase collagen types I and types III, fibronectin and TGF-β1 itself (Quaglino et al, 1990).

Wound healing in the TGF-β null transgenic mouse showed no difference in the rate of healing between transgenics and wild-type animals (Letterio et al, 1996). Differences were only seen when the inflammatory processes overwhelmed the healing processes at later times (Letterio et al, 1996). Immunohistochemistry revealed positive staining for TGF-β1 in granulation tissue of the wild-type animals but not the transgenic knock-out mice (Letterio et al, 1996); but interestingly, the opposite staining pattern was seen with TGF-β3. This suggests that TGF-β3 may substitute for the paracrine functions of TGF-β1. These data support another study that demonstrated antibodies to TGF-β1 and –2 or

the addition of TGF-β3 have reduced scarring in incisional wounds in rats (Shah et al, 1995). But the study performed in rats (Shah et al, 1995) (an animal that normally heals without obvious scar formation) is controversial to another study (Wu et al, 1997) that reported no differences in the healing of rabbit ear wounds between TGF-β3 and TGF-β2 isoforms. TGF-β3 was even demonstrated to induce the expression of TGF-β1 mRNA (Wu et al, 1997).

2.2.4 Estrogen and TGF-β

Estrogen levels have been linked to levels of TGF-β1 (Ashcroft et al, 1997). As females age, decreased estrogen levels have been associated with reduced TGF-β1 levels. The rate of cutaneous healing may be decreased, but there is also reduced scarring. Previous studies have shown TGF-β1 to improve cutaneous wound healing characterized by an increased healing rate or wound strength (Ashcroft et al, 1997; Beck et al, 1993; Pierce et al, 1989). Human studies compared young women (regular levels of estrogen and thus higher TGF-β1) to post-menopausal women (decreased estrogen, decreased TGF-β1) and observed that the older women had reduced re-epithelialization and reduced collagen deposition at day 7 post-wounding using 4mm punch biopsies (Assoian et al, 1983). These effects could be reversed by hormone therapy. The young women healed with signs of densely packed layers of collagen throughout the wound as opposed to the parallel organization of fibers seen in normal skin. The control of TGF-β1 levels by estrogen is a post-translational process since the use of cycloheximide has no effect (Ashcroft et al, 1997). *In vitro* testing of estrogen and progesterone showed inhibition of cellular proliferation of fibroblasts was independent of TGF-β1 (progesterone had

significantly greater inhibition) but the inhibition of mink lung epithelial cells was TGF-β1-dependent (with no effects seen using TGF-β2, or β3) (Ashcroft et al, 1997). Differences in cellular response between the *in vitro* and *in vivo* environments included the steady state levels of TGF-β1 mRNA that was increased *in vivo* and not in culture (Ashcroft et al, 1997). This observation could be explained by 1) other cells that are capable of secreting TGF-β1 migrating to the wound, 2) other estrogen-induced interactions *in vivo* or 3) differences in mRNA stability between the *in vivo* and *in vitro* conditions (Ashcroft et al, 1997).

2.2.5 TGF-β and Other Cytokines in Wound Healing

Other cytokines studied in wound healing include FGF, EGF and keratinocyte growth factor (KGF) (Clymer et al, 1996). FGF and EGF were observed to enhance wound healing. For example, FGF has been shown to increase closure rate and finally to produce a normal tissue architecture after topical applications to the tympanic membrane perforation model (Vrabec et al, 1994). FGF also stimulates the secretion of proteinases which lead to matrix remodelling (Clymer et al, 1996). Wound healing chambers implanted subcutaneously into the backs of rats were used to study the interaction between TGF-β1 and EGF. TGF-β caused an increase in collagen production, fibroblast proliferation and infiltrating inflammatory cells (Sporn et al, 1983). Chambers with EGF and TGF-β showed slightly higher levels of protein synthesis although these increases were not significant statistically (Sporn et al, 1983), yet *in vitro* work shows EGF to be antagonistic to TGF-β (Roberts et al, 1986). Direct subcutaneous injections of TGF-β into newborn mice caused angiogenesis and collagen production in fibroblasts which

was not observed with EGF (Roberts et al, 1986). KGF only produces a more organized wound repair process (Clymer et al, 1996). It promotes epithelial proliferation and differentiation, specifically the epithelial cells and not the mesodermal cells due to receptor specificity (Clymer et al, 1996). Enzymes associated with KGF include neutral proteinases (plasminogen activator and gelatinase) (Clymer et al, 1996).

Another cytokine investigated in wound healing is platelet-derived growth factor (PDGF) (Pierce et al, 1989). This study looked at the injection of recombinant TGF-β1 and PDGF (B-chain homodimers) into full thickness incisional wounds in rats. There was a marked increase in procollagen type I production in response to both cytokines (Pierce et al, 1989). TGF-β1 treated incisions showed increased wound strength over PDGF at 5 days after injury, but both cytokines responded similarly at 7 days post wounding. Whereas TGF-β1 did not accelerate wound healing after 2 weeks, PDGF was capable of more sustained enhancement of healing, but the mechanism is believed to be through the induction of increased TGF-β1 production. PDGF treated wounds showed increased breaking strength, fibroblast numbers, granulation tissue and TGF-β1 production. Increased procollagen type I immunostaining from PDGF may come from the increased fibroblasts attracted to the site of injury as opposed to actual increased cell output (no differences were seen with procollagen type III). This evidence suggests that the role of PDGF in wound healing is the recruitment of cells and its effects on enhanced healing comes from the induction of TGF-β1.

Results observed in wound healing models are probably not due to the action of TGF-β1 alone, but from the concerted action of exogenous TGF-β1 and the other

inflammatory cytokines released by recruited immune cells to the wound (Lin et al, 1995).

Although TGF-β is very important in wound healing, excessive collagen deposition can lead to undesirable conditions such as hypertrophic scarring and keloid formation. Therefore specific anti-TGF-β antibodies might used to reduce dermal scarring while maintaining the repair strength (Robey et al, 1987). The mechanism for this is believed to be through inhibition of the autoinduction of TGF-β. Therefore, because of the risks of excessive ECM deposition, TGF-β would be useful in situations where there is an impairment to wound healing, and antibodies to TGF-β could be used on normal individuals to reduce the amount of scarring.

2.3 TGF-β TRANSGENIC MODELS

2.3.1 Transgenic Animals

A very powerful tool available to a researcher is the generation of a transgenic animal. The effects of certain elements can be tested with the use of tissue cultures and cell lines, where examinations of both morphology and/or culture medium will provide any evidence of cellular response to stimuli. However, to assume the same results could be extrapolated to include whole organisms could be wrong. *In vitro* effects are much simpler than a response *in vivo* because of the vast number of cells present and the multitude of interactions present within an organism. Transgenic models are invaluable especially in studies of cytokines which usually have more than one function and affects more than one cell type. The testing of specific gene activities is possible through transgenic models (Thompson et al, 1993). Tissue histology, immune responses, and physiological effects can all be evaluated (Elias et al, 1996). They can define relationships between disease, normal responses, and alterations at the molecular level.

2.3.2 Developing Transgenic Animals

The first successful transgenic mouse was developed in 1980 (Ho et al, 1994). The most common technique for producing transgenic mice involves the initial creation of a 'transgene' construct. The transgene contains the gene of interest (in this case TGF-β) to be studied along with a chosen promoter sequence. The promoter can be used to target transgene expression to specific tissues. Examples of tissue-specific promoters include the albumin promoter for hepatocytes (Sanderson et al, 1995), CC10 or SP-C for lung

tissue (Elias et al, 1996), and keratin promoters for skin (Cui et al, 1995). Transfer of genetic information is by direct microinjection into the pronucleus of early blastocytes. Viable blastocytes are then transplanted into the oviducts of a chosen pseudopregnant female mouse (usually greater than 50 % of the injected blastocytes survive (Elias et al, 1996)). The natural course of pregnancy will generally provide only 10-40% of the progeny carrying the transgene that is integrated randomly and in varying copy number into the genomic DNA (Ho et al, 1994). The offspring that arise from the manipulated blastocyte is then referred to as the founder mouse and if the integrated DNA is stable, it will be passed along to its offspring. The performance of the construct will depend on variables such as copy number, the insertion site and the promoter construct.

2.3.3 Keratin 14 Promoter

The keratin 14 (K14) promoter is important for wound healing studies in transgenic animals. Keratinocytes provide mechanical protection against the external environment and also prevent water loss by differentiating into an impermeable stratum corneum (Eckert, 1989). Keratinocytes produce insoluble heteropolymeric proteins called keratins that are used as building blocks of epithelial intermediate filaments (Steinert and Roop, 1988). Injury will cause synthesis and secretion of growth and chemotactic factors for lymphocytes (Kupper et al, 1988), thereby initiating an immunological response. Keratin 14 and keratin 5 are specifically synthesized in the basal layer of stratified epithelium (mitotically active cells) (Ohtsuki et al, 1992; Tomic-Canic et al, 1992; Wang et al, 1997). The K14 promoter of the K14 gene is 2.2kb in size (Staggers et al, 1995) but the specific DNA sequences used for its regulation have not been identified (Tomic-Canic et

al, 1992). Transgenic models have shown K14 expression to be found primarily in the external epithelium (Wang et al, 1997).

2.3.4 Basement Membrane

The basement membrane is a natural barrier that separates the epidermis from the dermis and is composed of laminin, type IV collagen and heparan sulphate proteoglycans (Yorchenco et al, 1990). Protein expressed in the epidermis from keratin-promoted transgenes must pass through the basement membrane to exert effects in the dermis. Studies using a two chamber model with keratinocytes on top of a reconstituted basement membrane matrix showed that increased levels of cytokines could pass through the barrier with some loss from non-specific binding to the plastic surface but not to the membrane (Kondo et al, 1997). Therefore, keratinocyte products can cross into the dermis and initiate a biological response. Other studies have shown that proteins ranging in size of several kD to 180 kD can cross the basement membrane (Katz et al, 1994). Studies have only looked at IL-1α, IL-6 and TNF-α, but differences in cytokine glycosylation, 3-D structure and activation may affect each one differently in its stability and ability to cross the basement membrane to produce a biological effect in the dermis (Cebon et al, 1990; Pennica et al, 1984).

In the K14-TNF-α transgenic mouse described by Chieng et al (1992), they reported elevated serum levels of TNF-α, clearly demonstrating the ability of the transgene product to cross the basement membrane. They attributed morphological abnormalities such as cachexia, growth inhibition, intestinal and hepatic necrosis, graft-versus-host disease and death to the increased TNF-α. Detmar et al (1998) reported that their

transgenic mouse generated with the K14 promoter and the vascular endothelial growth factor (VEGF) produced features of chronic inflammation. Other abnormalities included mast cell accumulation and increased leukocyte recruitment to the sites of inflammation.

2.3.5 TGF-β and Transgenic Animals

The following section will cover experiments that used transgenic animals in the study of TGF-β. One experiment involved the use of a porcine TGF-β cDNA attached to an albumin promoter transgene construct to overexpress the cytokine in liver cells (Sanderson et al, 1995). Transgenic animals allowed the study of the effects of TGF-β upon multiple tissues and organs. Hepatic fibrosis and glomerulonephritis were seen in these animals. This transgenic model was also useful for the detection of extrahepatic lesions (involving the arteries of the heart and kidneys) caused by increased TGF-β. Activation of TGF-β can be through the exposure of the complex to acid or alkali conditions, urea, heat, or proteinases (Border et al, 1992; Gleizes et al, 1997; Lyons and Moses, 1990); but in this and other transgenic models, the transgene can be manipulated so that the cysteine residues at position 223 and 225 are replaced by serine. This will prevent the formation of the disulfide-bridge, destabilizing the LAP and therefore preventing its association with active TGF-β (Brunner et al, 1989; Cui et al, 1995; Samuel et al, 1993). An important property of transgenic models is that they allow systemic effects to be observed. In addition to the observed growth retardation, many of the mice died of renal failure within the first 4-6 weeks after birth although the expression of TGF-β1 was targeted to the liver.

In addition to secreting active TGF-β, transgenic mice can also produce inhibitors of TGF-β. Activation by plasminogen will induce cleavage of LAP from TGF-β; but apolipoprotein(a) expression in transgenic mice will inhibit this effect (Grainger et al, 1994). TGF-β inhibits proliferation of human vascular smooth muscle cells and it was hypothesized that indirect inhibition of TGF-β by apo(a) will lead to atherogenesis. Apo(a) is one of the risk factors for atherosclerosis and the level of apo(a) directly correlated with the amount of TGF-β inhibited which in turn produced fatty lesions in these animals. Transgenic models allow the study of one particular step in a pathway by manipulating the test conditions.

Transgenic models are essential to the studies relating TGF-β and homeostasis. Cui et al (1995) ligated simian TGF-β1 cDNA to a keratin 10 promoter to target its expression to suprabasal keratinocytes. They showed that TGF-β inhibited proliferation of keratinocytes *in vivo* as well as *in vitro*. By using bromodeoxyuridine (BrdU) as an indication of DNA synthesis, they revealed that there was surprisingly a 2-3 fold increase in epidermal DNA labelling indices without any histological evidence of hyperplasia in the untreated animals. When hyperplasia was induced in the mice by the addition of 12-tetradecanoyl-phorbol-13-acetate (TPA), the increased levels of TGF-β type II receptors coincided with the inhibition of cell proliferation. This suggests that TGF-β type II receptors are important to the homeostatic mechanisms of the epidermis and the anti-proliferative effect of TGF-β is through its interaction with this receptor. The lack of hyperplasia observed in the presence of increased BrdU labeling was explained by the action of TGF-β1 to induce terminal differentiation and apoptosis (Rotello et al, 1991)

which resulted in increased epidermal cell turnover and thus increased DNA labeling was just an effort to replace differentiating cells.

In addition to studying increased TGF-β, transgenic mice are equally important in observing the effects of the absence of TGF-β activity. The TGF-β transgene can be constructed so that it disrupts TGF-β1 gene function in the embryonic stem cell through homologous recombination (Kulkarni et al, 1993). The effects of the null genotype can be observed even *in utero* in that two-thirds of the embryos will die before birth. Interestingly, the ones who do proceed to full term appear normal at birth, but after 2 weeks they develop what is described as "wasting syndrome" and die after another 1-2 weeks. Post-mortem histological examination of these animals revealed many inflammatory lesions in the organs and especially the heart and lungs.

Autoimmune disorders is another area where TGF-β1 null transgenic mouse offers insight (Kulkarni et al, 1993). Lesions seen in TGF-β1 knockout mice are similar to those seen in graft vs. host disease and some viral inflammatory lesions (e.g. Kilham rat virus, minute virus of mice and Sendai virus), although the locations are different. It was deduced from these observations that the lack of TGF-β may somehow have increased the sensitivity of the host to autoantigens and the subsequent lethality (Gamble et al, 1988; 1979; Lindsey et al, 1991; Rappaport et al 1979). This demonstrates another important use of transgenic models; namely developmental studies that require studying specific elements over a long period of time.

Transgenic animals are not limited to the study of cytokines, they are equally important in cancer research. TGF-β1 may also play a role in cancer as shown by studies in malignant cells (Thompson et al, 1993). Levels of TGF-β1 mRNA are increased just

before tumor cells show exponential growth. In malignant mice and human prostate cells, there was also elevated TGF-β1 expression. The effects of TGF-β often depend upon the specific cell type involved. Malignant epithelial cells in culture secrete TGF-β1, and most malignant cell lines have lost their ability to respond to TGF-β1. Exceptions are rat prostate epithelial cells which have been shown to be stimulated to proliferate by TGF-β1 even when transformed (Shain et al, 1990).

Although the benefits of transgenic mouse models have been stressed, there are still some limitations to their use. Firstly, the promoter is critical to transgene function and effectiveness. If the promoter chosen is constitutively active, continuous expression of the transgene would result. This would make studies on the responsiveness of these genes to different stimuli impossible. Another major disadvantage to transgenic models in mimicking human diseases is that many malignancies develop over years or decades which makes chronic studies impractical and expensive.

2.4 TGF-β AND INTERFERON-γ

2.4.1 Introduction to Interferons

Although once described as antiviral agents, interferons (IFNs) are now known to be integral to immune response and its modulation. Of the five IFNs (IFN-α, IFN-β, IFN-γ, IFN-δ, and IFN-ζ) which are classified by differences in biological activities, gene structure and sources, IFN-γ is the most extensively studied. It consists of a 34kDa homodimer glycoprotein held together through non-covalent bonds. Sources of IFN-γ include T lymphocytes and natural killer (NK) cells in comparison to monocytes and fibroblasts which secrete IFN-α and IFN-β respectively.

2.4.2 Functions of IFNs

The most dramatic function of IFN is in the protection of the host against viruses. Experimental administration of anti-IFN-α or β antibodies increased the susceptibility of mice to viral infections (Gresser et al, 1990). These antibodies were also used to show that suppression of IFNs will enhance proliferation of tumors in mice; thus supporting the idea that IFNs possess antiproliferative effects.

IFN-α may be more important in viral infections, but IFN-γ is critical to the regulation of the immune system. Its most important role may be in the induction of expression of major histocompatibility complex class I and II antigen on the surface of immune cells (Lindsey et al, 1991). Through its interaction with interleukins (ILs) and other cytokines, IFN-γ is believed to be involved in many immunological responses; some of these

include nitric oxide production (Lin et al, 1995) and reduced NK mediated cytolysis through the MHC class I antigen (Ma and Niederkorn, 1995).

2.4.3 Interactions Between TGF-β and IFN-γ

Similar to TGF-β, IFN-γ has a variety of different effects depending upon the target cell. In normal epidermal keratinocytes, it will cause squamous differentiation (Saunders et al, 1994). TGF-β possesses the ability to suppress squamous cell-specific genes but the cells are still able to proceed to a state of quiescence (normally preceded by squamous differentiation). This evidence implies that TGF-β and IFN-γ work by different pathways. The ability of IFN-γ to disrupt intestinal epithelium *in vitro* can also be reduced by TGF-β1 (Planchon et al, 1994). The effects of TGF-β1 include dose-dependent inhibition of IFN-γ lasting up to several days after a single exposure. By increasing IFN-γ concentrations, even these could be abrogated (Planchon et al, 1994). In microglial cells and peritoneal macrophages, IFN-γ induced increased Fc receptor and MHC class II expression. When the cells were exposed to both IFN-γ and TGF-β, the expression of these molecules was suppressed. TGF-β alone reduces MHC class II only on macrophages but decreased Fc receptors can be seen in both cells.

An important function of NK cells is the destruction of tumor cells which shows an inverse correlation with expression of MHC class I molecules (Ma and Niederkorn, 1995). Exposure of ocular melanoma cells to TGF-β can enhance NK cell cytolysis by 17% due to a reduction in MHC class I molecules of 52-62%. IFN-γ is known to increase MHC expression, and thus, by exposure of the same cells to IFN-γ, the amount of MHC

class I antigen increased to 80% and the cytolytic activity dropped to less than 10%. Therefore, TGF-β may be important in increasing the susceptibility of tumors to the natural host immune response through modulation of MHC class I antigens.

TGF-β can also inhibit MHC class II antigens (surface proteins), mRNA and promoter activity as demonstrated by studies of astrocytes (Brouty-Boye and Zetter, 1980). Thus, both cytokine-induced and constitutive gene expression would be inhibited. TGF-β does not affect the expression of other genes mediated by IFN-γ and intracellular signaling through tyrosine phosphorylation. Thus, modulation of MHC class II genes by TGF-β may be by indirectly regulating factors which subsequently interact with the MHC gene.

Other cytokines are equally important to the expression of MHC class II antigen. One important factor in the initiation of any immune response is the activation of macrophages into antigen presenting cells (APCs) characterized by increased expression of MHC class II antigen (Gollnick et al, 1995). IFN-γ and IL-4 can induce class II antigens on B lymphocytes and macrophages. Interestingly, TGF-β antagonizes IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF), and was actually shown to enhance the effect of IFN-γ in inducing expression of these genes. Nuclear run-on assays revealed regulation by TGF-β at the transcriptional stage for IFN-γ, but IL-4 and GM-CSF were inhibited post-transcriptionally (Gollnick et al, 1995).

Retinal pigment epithelial cells provided more evidence that TGF-β1 and -2 antagonize IFN-γ (Gabrielian et al, 1994). The induction of MHC class II antigens on these cells was inhibited by TGF-β1 or -2. The protein kinase C (PK-C) pathway appears to be involved in inhibition of IFN-γ by TGF-β because it was shown that when PK-C

activators were used in conjunction with TGF-β1 or -2, the inhibition seen was stronger than with either alone.

TGF-β and IFN-γ could also work together during an immune response. Prior exposure of macrophages to TGF-β can increase nitric oxide (NO) secretion in these cells by increasing expression of nitric oxide synthetase mRNA (Lin et al, 1995) on subsequent treatment with IFN-γ. IFN-γ plus either ≥100 U of tumor necrosis factor-alpha (TNF-α) or ≥100 ng of lipopolysaccharide increased NO production by 85% or 27% respectively if the macrophages were first primed by TGF-β. TGF-β alone normally inhibits NO production (Wahl, 1994). In macrophage cultures, TGF-β1 also increased the mRNA for TNF-α production (Lin et al, 1995).

The pairing of TNF-α and TGF-β or IFN-γ has been used in other experiments. TNF-α normally inhibits cell lines from monocytic leukemias or oral squamous carcinomas by reducing the amount of c-myc mRNA (Hori et al, 1994). TNF-α plus TGF-β or IFN-γ could inhibit the proliferation of these monocytic or carcinoma cell lines. This inhibition by TNF-α was dose-dependent, regulated at the transcriptional level (normally steady-state RNA) and independent of protein synthesis (no effect of cycloheximide (Hori et al, 1994)).

IFN-γ has been shown to be effective in controlling parasitic diseases such as that caused by *Leishmania amazonensis* (Barral et al, 1995). TGF-β would inhibit this effect of IFN-γ in the macrophages, which in turn could be prevented by high levels of TNF-α (100 IU/mL) (Barral et al, 1995). Thus, it is likely that secretion of TGF-β from macrophages and other immune cells could promote growth of parasites by antagonizing

IFN-γ function. IFN-γ can also aid in the resistance of an organism towards *Toxoplasma gondii* infection (Hunter et al, 1995). In this model, IFN-γ is induced by increased IL-12 in combination with TNF-α or IL-1-β. In infected animals, there was both increased TGF-β transcripts and protein (Hunter et al, 1995). It was concluded that TGF-β suppressed the ability of IL-12 to induce IFN-γ production, and thus may be involved in T-cell independent resistance to *T. gondii*.

2.5 EFFECTS OF TGF-β ON Th_1 and Th_2 CELLS

2.5.1 Introduction to Th_1 and Th_2

The human immune system is centered around recognition of antigens and inducing a response through the activity of specific cells (e.g. $CD8^+$ Cytotoxic T-Cells, NK cells) or promoting the development of B cells into antibody secreting plasma cells. Central to either pathway is the $CD4^+$ T-helper (Th) cell whose main functions are to interact with APCs via MHC antigens and $CD4^+$ receptors and to secrete different cytokines to induce a response by effector cells.

There are three $CD4^+$ cell subsets. Th_1 cells primarily produce interleukin-2 (IL-2), interferon-γ and tumor necrosis factor (TNF) (Liblau et al, 1995; Romagnani, 1995). These cells support macrophage activation, are involved in delayed-type hypersensitivity (DTH) reactions, immunoglobulin isotype switching to favor IgG-2a (important for opsonization and complement fixing), antibody-dependent cytotoxicity, and are vital to the defense against intracellular pathogens (Liblau et al, 1995; Mossman and Coffman, 1989; Romagnani, 1984; 1995; 1996). Th_1 cell functions are described as phagocyte dependent host responses whereas Th_2 functions are phagocyte-independent (Romagnani, 1996).

Th_2 cells produce IL-4, -5, -6, -10 and –13 (Liblau et al, 1995; Romagnani, 1996). These cells promote optimal humoral response by providing help for B-cell activation (Liblau et al, 1995; Romagnani, 1995; 1996). They induce immunoglobulin class switching to IgG-1 and IgE (Liblau et al, 1995; Romagnani, 1996). Th_2 cells are important for mucosal immunity through facilitating IgA synthesis and the growth and

development of eosinophils and mast cells (Romagnani, 1996). IL-4, -10 and -13 also work to inhibit macrophage functions (Mossman and Coffman, 1989; Romagnani, 1994). Th_0 cells are described as precursors to Th_1 and Th_2 cells because they possess a cytokine profile similar to both subsets (Liblau et al, 1995). IFN-γ will inhibit differentiation and effector functions of Th_2 cells while simultaneously promoting Th_1 subsets, whereas IL-4 will direct Th_2 development (Liblau et al, 1995; Romagnani, 1995). IL-12 also induces Th_1 development (Hoehn et al, 1995).

2.5.2 Differentiation of Th_1 and Th_2

When these cells are first stimulated and thus not yet differentiated, IL-2 is the primary cytokine secreted (Sad and Mossman, 1994). When the cells differentiate into Th_1 and Th_2 subsets after a second stimulation, a different cytokine patterns appear. Although the two subsets possess different cytokine profiles, they are still able to influence each other (Li et al, 1993). Cross regulation is seen as Th_1 secretion of IFN-γ can suppress the secretion of IL-10 from Th_2 cells and vice versa.

TGF-β is one of the most potent anti-proliferative cytokines for T and B cells (Li et al, 1993; Kulkarni et al, 1993). TGF-β mRNA is increased after the activation of these cells (Rossi et al, 1988) and is also shown to prevent the differentiation of these cells (Van Obberghen-Schilling, 1988). Sad and Mossmann (1994) showed that TGF-β prevented T-cells from secreting cytokines normally associated with Th_2 cells, namely IL-4 and IL-5. Th_1 development was inhibited by using anti-IFN-γ antibodies. The use of both TGF-β and anti-IFN-γ kept T-cell populations at undifferentiated stages, shown by consistent

IL-2 secretion after re-stimulation and the lack of IFN-γ or IL-4 (Sad and Mossmann, 1994).

It has also been shown that TGF-β can favor one subset over the other. Exposure of Th_2 cells to TGF-β would increase production of IL-10, thereby shifting the balance between Th_1 and Th_2 subsets to favor Th_2 (Maeda and Shiraishi, 1996). The anti-TGF-β antibodies reduced Th_2 function (indicated by decreased IL-4 levels) and restored the suppressed Th_1 cytokine production (Hori et al, 1994).

2.5.3 Th_1 Stimulation by TGF-β

Th_1 development that was stimulated by TGF-β2 required a high concentration of IL2 (Hoehn et al, 1995). In this study, strains of mice were distinguished by either TGF-β2 promotion (strains C57B1/6, BALB/c, NMR1) or inhibition (C3H/He, CBA/J) of Th_1 development. Th_1 cells were still inhibited after IL-12 was added to the cells. To activate the cells, anti-CD3 monoclonal antibodies were used. The next step required hybrids of $CD4^+$ cells (C57B1 and CBA/J). When these cells were activated with only anti-CD3 antibody, TGF-β inhibited the Th_1 phenotype, but induced Th_1 development when costimulated with CD28 antibody. Studies on the IL-2 levels correlated with Th_1 development; levels were low after a single activation with anti-CD3, but high after co-stimulation. IL-2 was only partially suppressed in the presence of TGF-β and anti-CD28 antibodies. Addition of exogenous IL-2 and TGF-β2 enhanced Th_1 development while the removal of IL-2 is sufficient to inhibit Th_1. Thus, it could be implied that Th_1 development is also determined by the ability of the $CD4^+$ cells to produce IL-2.

In addition to IL-2, IL-12 and IFN-γ are essential to the development of $CD4^+$ cells into Th_1 subsets (Spaccapelo et al 1995). Studies involving the Th_1 effector cells during infections caused by *Candida albicans* suggest that TGF-β is another cytokine which is important in Th_1 development and subsequent resistance to the yeast. Comparisons of infected and vaccinated mice showed TGF-β levels were significantly lower in the non-vaccinated mice (Spaccapelo et al 1995). Anti-TGF-β antibodies used in the vaccinated animals produced a loss of resistance and thus made them susceptible to infection. Also noticed in these mice was a decrease in IL-12 and IFN-γ levels along with a rise in IL-4 and IL-10 (Spaccapelo et al 1995). Thus, resistance is not only from Th_1 expression, but TGF-β may be necessary to prevent a shift from the Th_1 subset to Th_2.

2.5.4 Th_2 Apotosis delayed by TGF-β

Th_2 cells are responsible for the development of B cells into antibody secreting plasma cells as demonstrated by Zhang et al (1995) who took $CD4^+$ cells just before differentiation into effector Th_2 cells after an initial stimulus with antigen. These cells normally mediate their function by interacting with APCs and secreting cytokines. With a second exposure to antigen, the cells proliferate and secrete high concentrations of cytokines; without it, after 4-7 days, these cells undergo apoptosis. Apoptosis can be partially blocked by IL-2 or TGF-β alone. IL-2 also promoted proliferation induced by antigen, whereas TGF-β had no observed effect on proliferation. When IL-2 and TGF-β were given together, the results were dramatic and synergistic. Th_2 specific effectors were greatly increased and the development to this subset was prolonged.

2.6 SUMMARY AND HYPOTHESIS

TGF-β is interesting to study not only for its roles in wound healing and immune responses, but at a genomic level as well. It has a unique promoter with many regulatory sites. In addition to its negative regulatory elements and enhancer regions, there are two major transcriptional start sites (Kim et al, 1989). The key to TGF-β's ability to amplify its own signal by autoinduction is also believed to be located in two areas, one upstream from the start site of transcription and another between the two promoters (Kim et al, 1990).

Transgenic animal models have been invaluable in research on TGF-β. They allow almost simultaneous study of the roles TGF-β plays in wound healing, immune modulation, and other physiological mechanisms (Elias et al, 1996). Hepatic fibrosis and glomerulonephritis were observed in transgenic animals with over expression of TGF-β targeted to the liver (Sanderson et al, 1995). Also in these animals, extrahepatic lesions in the heart and kidneys were indications that the effects of TGF-β were not localized (Sanderson et al, 1995). In addition to the effects of excessive TGF-β, models of decreased TGF-β serum levels could also be developed. Mice born with the null genotype developed 'wasting syndrome' and died as a result of inflammatory lesions in many organs (Kulkarni et al, 1993).

TGF-β has been implicated in many immune functions. It generally suppresses the differentiation and proliferation of T and B lymphocytes (Roberts et al, 1993). TGF-β also works antagonistically to inflammatory cytokines such as TNF and IFN-γ (Roberts et al, 1993). Loss of TGF-β activity can give rise to basal cell proliferation or suprabasal

hyperproliferation (Glick et al, 1993). There have also been reports that TGF-β levels may be important to the development of tumors because of its immunosuppressive effects on $CD4^+$ Th cells (Li et al, 1993). Thus, the anti-proliferative effect of TGF-β on various cell types is complex and not easily understood.

Inhibition of MHC class II surface antigens, mRNA and promoter activity by TGF-β (Brouty-Boye and Zetter, 1980) has also been observed. It worked antagonistically to IL-4 and GM-CSF in the induction of MHC class II antigen expression but enhanced IFN-γ activity in macrophages (Gollnick et al, 1995). Experiments with retinal epithelial cells revealed TGF-β antagonized the effects of IFN-γ in the induction of MHC class II antigens (Gabrielian et al, 1994). Therefore, the targeted cell type is a very important element in the study of TGF-β and general statements made could be false. TGF-β works together with many cytokines to modulate immune responses.

Th_1 and Th_2 subsets of $CD4^+$ cells have different responses to TGF-β. TGF-β prevents the proliferation of T and B cells (Kulkarni et al, 1993; Li et al, 1993). Normally, IFN-γ will promote the development of Th_1 cells, but the introduction of anti-IFN-γ antibodies and TGF-β could keep Th cell populations undifferentiated (Sad and Mossman, 1994). TGF-β prevented the secretion of IL-4 and IL-5, cytokines important to the development of Th_2 subsets (Sad and Mossman, 1994). Conflicting evidence was seen when experiments revealed TGF-β increased the secretion of IL-10 by Th_2 cells and promoted the Th_2 development over Th_1 (Maeda and Shiraishi, 1996). Anti-TGF-β antibodies could restore Th_1 cytokine development (Hori et al, 1994). Other studies showed that TGF-β could also stimulate Th_1 development, but only if high levels of IL-2 were also present (Hoehn et al, 1995).

The role TGF-β plays in ECM formation may be the answer to controlling fibrosis. TGF-β promotes matrix protein synthesis by increasing their levels of steady-state RNA (Van Obberghen-Schilling, 1988; Varedi et al, 1995) while inhibiting proteinases (Varedi et al, 1995). The most dramatic effect of TGF-β is upon fibroblasts. Fibroblasts are one of the few cell types that TGF-β will induce to proliferate (Rossi et al, 1988). At the site of injection into skin, TGF-β will induce granulation tissue (Roberts et al, 1993).

TGF-β is important to many aspects of wound healing. It inhibits proliferation in epithelial cells, endothelial cells and lymphocytes (Bronzert et al, 1990; Van Obberghen-Schilling, 1988) but has opposite effects on fibroblasts (Rossi et al, 1988). Its exact role in hypertrophic scarring is becoming clearer. With better understanding of the roles TGF-β plays, we may be able to control this disorder and many others characterized by excessive fibrosis.

Hypertrophic scars develop after a prolonged inflammatory stage after injury and are characterized by excessive extracellular matrix production. TGF-β is known to be fibrogenic and released after injury by platelets and inflammatory cells. Its interactions with different cytokines and immunomodulary effects may play a large part in wound healing. Previous wound healing experiments have been performed using incisional wounds with exogenous TGF-β administered directly to the dermis but the role of TGF-β produced by the keratinocytes has not yet been elucidated. Re-epithelialization of wounds is an important aspect of wound healing and therefore TGF-β produced by migrating keratinocytes can not be ignored. The development of a transgenic animal that limited the production of excessive TGF-β1 strictly to the keratinocytes in the epidermis would enable us to study the role of TGF-β in wound healing. It is our goal to evaluate the

effects of TGF-β from keratinocytes on wound healing. In order to mimic a more natural physiological response, use of a transgene that produces a latent form of TGF-β is necessary which would also permit study of its activation. It is our hypothesis that TGF-β1 produced by the keratinocytes during wound healing plays a role in the development of hypertrophic scarring.

CHAPTER THREE

MATERIALS AND METHODS

3.1 TRANSGENE CONSTRUCTION AND THE PRODUCTION OF TRANSGENIC ANIMALS

The keratin-14-TGF-β1 construct (Figure 1) used to produce the transgenic line of mice was first constructed and tested *in vitro* as described by Ghahary et al (1998). The pG3Z:K14 expression vector contains the keratin 14 promoter so that when the gene of interest is cloned into the vector, transcription is targeted to keratinocytes. There is a BamH1 restriction site between the promoter and polyadenlylation site in which the cDNA of human TGF-β1 could be inserted. Blunt-ended ligation between the expression vector and the cDNA for TGF-β1 was required because of the presence of a BamH1 restriction site within the coding region of TGF-β1. The expression vector was cut with BamH1 and the TGF-β1 cDNA was cut with Pst I and EcoR1, with the overhanging ends filled in with the Klenow fragment. After blunt-ended ligation, the construct was designated pG3Z:K14-TGF-β1 and tested *in vitro* (Ghahary et al, 1998).

Transgenic animals were produced by the microinjection of the keratin-14-TGF-β1 construct into fertilized mice embryos prior to the transplantation into pseudopregnant mice as described by Gordon et al. (1980). Fertilized, single-cell embryos from random FVB/N x FVB/N matings were harvested 16 hours post-coital. These embryos were then microinjected with roughly 400-1000 copies of the K-14-TGF-β1 construct. Any surviving embryos (still in the single-cell stage) were transplanted into pseudopregnant

females. The procedure was performed by the University of Alberta Transgenic Mouse Facility, University of Alberta.

3.2 DNA ISOLATION

Tail biopsies (0.5-1.0 cm) were minced on ice with a scalpel then placed in 350 ul of DNA extraction solution (0.005 M Tris buffer at pH 8.0; 0.025 M EDTA at pH 8.0; 0.05 M NaCl; 0.005% SDS). This mixture was then homogenized (Ultra-Turrax T25, Janke and Kunkel IKA-Labortechnik at 13500 rpm) and 30 uL of proteinase K added (Boehringer Mannheim) prior to samples being incubated at 52°C overnight. 6M NaCl was used to precipitate proteins from the samples which were then centrifuged to collect supernatant. An equal volume of isopropanol was added to precipitate the DNA at –20°C. After a minimum time of 2 hours, samples were centrifuged at approximately 14900 x *g* for 20 minutes; pellets were washed once with cold 70% ethanol, centrifuged for 5 minutes and all traces of ethanol removed. The pellets were redissolved in TE8 buffer consisting of 10mM Tris-HCl (pH 8.0) and 1mM ethylenediamine tetraacetic acid. Concentrations were calculated based upon the absorbance of each sample at 280 nm.

3.3 SOUTHERN BLOTTING

For detection of the transgene, 10 ug of DNA for each sample was digested with BamH1 (GibcoBRL) at 37°C for at least 6 hours. Restriction products were run on 1% agarose gels (GibcoBRL) in 1X TBE buffer. Gels were fractionated under UV light, placed in a denaturing solution (0.5M NaOH, 1.5M NaCl) and then neutralized (1M Tris, pH 8.0; 1.5M NaCl) prior to transferring onto nitrocellulose (Micron Separations

Incorporated, Westboro, MA) by capillary action. Membranes were baked under vacuum at 80°C for 2 hours prior to hybridization. ^{32}P-labelled DNA probes were made using a nick translation kit (Amersham LIFE SCIENCES, Piscataway, NJ) substituting α-^{32}P-dCTP for the dCTP. Passing the reaction mixture through a column filled with Sephadex beads (Sephadex® G-50 Medium, Pharmacia Biotech) separated the probe from unbound nucleotides. All blots were incubated at 42°C for at least 2 hours in prehybridization buffer (6X SSC, 10X Denhardts, 0.5% SDS, 100ug/mL salmon-sperm DNA, 50% formamide) and probed overnight. Blots were washed first in a 2X sodium chloride/citrate buffer (1X=0.15M sodium chloride, 0.015M sodium citrate, pH 7.0) and 0.1% sodium dodecyl sulfate solution at room temperature, then in a 0.2X sodium chloride/citrate and 0.1% sodium dodecyl sulfate solution at 60°C. Blots were subsequently exposed to X-ray film (BioMax MS, KODAK) from 1 to 24 hours at –80°C.

3.4 EXTRACTION OF RNA FROM SKIN AND WOUNDS

Tissue was collected in 4 M guanidinium isothiocyanate and snap frozen in a dry-ice and ethanol mix and processed using the procedure of Chomczynski and Sacchi (1987) for isolation of RNA. Samples were homogenized using either a Teflon pestle (Fisher) (wounds harvested from wound healing experiment) or homogenizer (Ultra-Turrax T25, Janke and Kunkel IKA-Labortechnik at 13500 rpm) (organs collected for PCR) before the addition of 0.1 volume of 2M sodium acetate. The solution was vortexed to ensure proper mixing prior to the addition of 1.1 volume of phenol and 0.2 volume of chloroform-isoamyl alcohol (49:1) solution to remove unwanted cellular debris and proteins after centrifugation at 14900 x *g* for 20 minutes. An equal volume of

isopropanol was added to the aqueous layer and this mixture was kept at –20°C to precipitate the RNA. Samples were spun down at 12100 x *g* for 30 minutes and the pellet redissolved in GITC. A second precipitation was performed with isopropanol and the RNA washed with 70% ethanol. RNA was dissolved in TE8 buffer and the concentration of each sample was determined from the absorbance at 260nm.

Samples of RNA for RT-PCR were treated with DNAse (GibcoBRL) after the second precipitation. Pellets were dissolved in DEPC-treated water and mixed with 10X DNAse buffer (1.0M sodium acetate and 50mM $MgCl_2$, pH 5.5), RNAse inhibitor (GibcoBRL) and DNAse then incubated at 37°C for 10 minutes. Phenol-chloroform extraction (as described earlier) was performed twice to remove all traces of DNAse before the precipitation of RNA with an equal volume of isopropanol at –20°C.

3.5 REVERSE TRANSCRIPTION

Using 2μg of total RNA for each sample, 500nM of antisense primer (human or mouse TGF-β1) and water were added to a final volume of 10μl. This mixture was incubated at 70°C for 10 minutes to denature the RNA, then 35°C for 1 minute for primer annealing and then back at 70°C for 1 minute to maintain the RNA single-stranded. Final conditions for Superscript™ enzyme (Gibco BRL) was as follows: 100U Superscript™ enzyme, 1X first strand buffer (50mM Tris-HCl, pH 8.0, supplied with enzyme), 0.5 μM dNTP mix, 0.01M DTT (supplied with enzyme), 0.05μg/μL BSA, and 2U RNAse inhibitor. Reverse transcription cycle was first incubation at 45°C for 90 minutes followed by 95°C for 3 minutes. Samples were put immediately on ice after cycle completion.

3.6 POLYMERASE CHAIN REACTION

Primers used for human TGF-β1 (5'-TAC GCC AAG GAG GTC ACC C-3' sense and 5'-CCG TTG ATG TCC ACT TGC-3' antisense) and mouse TGF-β1 (5'-GCT GAC CCC CAC TGA TAC GCC T-3' sense and 5'-GTT GTG TTG GTT GTA GAG GGCA-3' antisense) were synthesized by the Nucleic Acid Research Center, University of Alberta. PCR was performed on cDNA produced by reverse transcription of RNA samples (as described above). Paraffin wax (Paraplast X-tra, Oxford Labware) made into beads was used for a 'hot start' to minimize non-specific annealing of primers to cDNA. The lower mixture consisted of, 1X PCR buffer (Gibco BRL), autoclaved distilled water, 1.5 mM $MgCl_2$, 0.1mM dinucleotidetriphosphates (dNTPs) and 500nM of each primer (sense and antisense). One paraffin wax bead was melted at 80°C and allowed to cool at room temperature to produce a barrier preventing mixing of cDNA and primers. The top mixture consisted of sample cDNA, 1X PCR buffer and 2.5 units of Taq polymerase (Gibco BRL). The optimal PCR cycle involved denaturing at 97°C, annealing at 60°C and extension at 73°C for 35 cycles. PCR products were run on a 1% agarose gel with a 100 base pair ladder. Human TGF-β (hTGF-β1) product was 404 bp and the mouse TGF-β1 (mTGF-β1) was 483 bp. The positive control was plasmid TGF-β and the negative control was skin RNA from homozygous animals not reverse-transcribed.

3.7 QUANTITATIVE ENZYME IMMUNOASSAY FOR TGF-β1

Serum was collected by cardiac puncture after each mouse was euthanised by CO_2 asphyxiation. Blood was drawn into a sterile 3cc syringe with a 19.5 gauge needle

(Beckton Dickinson), then quickly transferred to a sterile 1.5 mL eppendorf tube (Fisher). Samples were left overnight at 4°C and spun 10 minutes at 790 x *g* to remove cells. Serum was removed and stored at –80°C until tested. Assays of serum samples were performed using kits purchased from R&D Systems (Quantikine, R&D Systems) and following package inserts. All serum samples collected for the immunoassay were diluted by a factor of 105 in the RD6M diluent supplied with each kit prior to the assay. Serum TGF-β was activated by the addition of 0.1 mL of 2.5N acetic acid/10M urea solution followed by neutralization with 0.1mL of 2.7N sodium hydroxide/1M Hepes to the diluted samples. The assay uses microplates precoated with the TGF-β type II receptor. Standards of recombinant human TGF-β1 and samples (0.2 mL/well) were added to the supplied microplates. After an incubation period of 3 hours at room temperature, excess standards and serum samples were washed with the provided washing buffer. Polyclonal antibody against TGF-β1 conjugated to horseradish peroxidase followed the first wash and was incubated for 1.5 hours at room temperature. The plate was washed again before the addition of the substrate solution which consisted of equal volumes of hydrogen peroxide and the chromogen tetramethylbenzidine (supplied with kit). After a 20 minute incubation of the substrate solution, the color development reaction was stopped by the addition of 50μL of 2N sulfuric acid. The plates were read immediately at 450nm and concentrations of samples were determined from the standard curve.

3.8 WOUND HEALING MODEL

All animal studies were conducted in compliance with Canadian Council on Animal Care (CCAC) guidelines and the University of Alberta Health Sciences Animal Policy and Welfare Committee regulations. Four full thickness 4mm skin biopsy wounds were placed on the dorsal surfaces of mice to observe differences in wound healing between mice homozygous for the K14-TGF-β transgene, heterozygous and normal (FVB Strain, Techonics). Mice were anaesthetized by the inhalation of metafane and the dorsal surfaces were depiliated by first shaving, then applying hair removing cream (Nair™). Punch biopsies were made and the skin collected for extraction of RNA into GITC, hydroxyproline analysis, and immunohistochemistry into 4% paraformaldehyde. At days 5, 7, and 9 post-wounding, 10 animals from each group were euthanised by CO_2 and so 40 wounds were evaluated for each group at each day by visual assessment for re-epithelialization. Serum was collected by cardiac puncture and wounds were harvested using a 6mm biopsy punch. Samples for immunohistochemistry were collected in 4% paraformaldehyde, tissues for RNA analysis were collected into GITC and snap frozen along with samples for hydroxproline in a dry ice-ethanol mix.

Acetate tracings of the wounds were also made prior to sample collection and these were scanned into a computer for analysis. Due to the limited resolution of the scanner and the wounds being too small to detect any differences in the rate of re-epithelilization from the tracings, the only definite information that could be extracted was confirmation of the visual assessment of wounds being open or closed.

3.9 PAI/L ASSAY FOR *IN VIVO* TGF-β DETERMINATION

Several animals were used for the measurement of TGF-β in the wounds using the plaminogen activator inhibitor-1/luciferase (PAI/L) assay as described by Yang et al (1999). Briefly, on specified days after wounding, all four full-thickness punch biopsy wounds were harvested using a 6mm punch, stacked, embedded in methyl cellulose, frozen in a dry-ice-ethanol bath and stored at –80°C. Sections were cut at a thickness of 20μm using a cryostat and placed onto round coverslips. The mink lung epithelial cells (MLECs) transfected with the PAI/L construct (truncated TGF-β-inducible plasminogen activator-1 promoter fused to a luciferase reporter gene) were a generous gift from Dr. Daniel Rifkin (New York University Medical Center, NY, New York). These cells were plated onto 24-well plates. Coverslips carrying the tissue sections were incubated in contact with the MLECs and luciferase activity detected using a scintilation counter (Beckman LS6000IC). Sample values were read off standard curves generated using recombinant TGF-β. Active TGF-β can be detected using this procedure. Total TGF-β required acid activation of the sections prior to assay by the addition of 3N HCl to a pH of 4.0 then neutralization using a 1.4N NaOH/ 0.7M HEPES solution to pH 7.4.

3.10 NORTHERN BLOTTING

Each sample of RNA (10 ug) was placed in a denaturing solution (formamide, MOPS, formaldehyde) and incubated at 65°C for 30 minutes prior to loading onto a 1% agarose gel. The RNA was transferred to a nitrocellulose membrane through capillary action and dried at 80°C under vaccuum. Probes were made as described under SOUTHERN BLOTTING and membranes were first incubated in a prehybridization solution prior to

probing overnight at 42°C. The blots were washed the next day in high, then low salt solutions before exposure to X-ray film (KODAK).

3.11 IMMUNOHISTOCHEMISTRY

Tissue sections for immunohistochemistry were collected into 4% paraformaldehyde before being processed and embedded in paraffin wax. Sections were cut at a thickness of 5μm and placed onto regular glass slides for staining with hematoxylin and eosin (H&E) or by the Masson's trichrome method. Sections for immunohistochemical detection of TGF-β were placed onto Aptex (3-aminopropyl-tetraethoxysilane, SIGMA) coated slide. Sections were heated to 60°C, dewaxed in xylene and then rehydrated. Slides were first submerged in a methanol-30% hydrogen peroxide mix (2:1) for 6 minutes, blocked with PBS containing 10% normal goat serum and 5% BSA (Sigma) for 30 minutes at room temperature, and then incubated overnight at 4°C with the anti-TGF-β polyclonal antibody IgG (Santa Cruz) diluted 50 times in PBS. After washing in PBS, the secondary antibody, biotinylated anti-rabbit IgG (VECTOR Laboratories Inc.) diluted 200 times in PBS and incubated 40 minutes at room temperature. A substrate solution consisting of 5% BSA, solutions A and B from a VECTASTAIN ABC kit (Dimension Laboratories Inc.) and PBS was next (60 minutes at room temperature), followed by incubation with DAB (3,3'-diaminobenzidine, SIGMA) (3 minutes at room temperature). For improved contrast, sections were countered stained in hematoxylin (Fisher) before dehydrating and mounting (Permount, Fisher Scientific).

3.12 HYDROXYPROLINE ANALYSIS

Tissue samples collected using biopsy punches (4mm for day 0; 6mm for day 5,7, and 9) were weighed wet, freeze dried and then weighed dry. Dried samples were hydrolysed by adding 1 mL of a 6N HCl and heated at 110°C for 22 hours. Distilled water (2mL) was then added and the samples frozen at –80°C before freeze drying overnight. Samples were redissolved in 1 mL of a 1mM HCl solution and centrifuged twice, discarding the pellet each time to remove any debris. Samples were then stored at –80°C until analyzed.

The colorimetric method for hydroxyproline analysis was performed as described by Stegemann and Stalder (1967). Hydrolysates were diluted 20 times in water. Oxidant solution consisted of 0.42M sodium acetate, 0.13M trisodium citrate-dihydrate, 0.026M citric acid and 38.5v/v isopropanol. To 500 uL of diluted hydrolysate, isopropanol (1mL), working oxidant (1:4 solution of 7% chloramine T: oxidant) (0.5mL) and Ehrlich's reagent (1mL) was added respectively, with vortexing between each addition and incubated at 60°C for 21 minutes and at room temperature for 60 minutes to complete the color development. All reactions were performed in triplicate. Dilutions of pure hydroxyproline solution were used to generate a standard curve. All samples were read at 562nm.

CHAPTER FOUR

RESULTS

4.1 *IN VITRO* TESTING OF THE pG3Z:K14-TGF-β1 CONSTRUCT

The plasmid pG3Z:K14-TGF-β1 was tested *in vitro* for proper functioning as described by Ghahary *et. al* (1998). Northern analysis of transfected cells showed increased expression of TGF-β1 mRNA specifically in keratinocyte lines. TGF-β1 protein levels were significantly increased in the culture medium of transfected cells as evaluated by enzyme-linked immunosorbent assay. Mink lung epithelial cell growth inhibition assay revealed an increased TGF-β activity from the modified keratinocytes and co-culture systems of keratinocytes and fibroblasts showed that the TGF-β produced by the keratinocytes stimulated collagen production in the fibroblasts. These results show that the K14-TGF-β construct is properly expressed in keratinocytes but not in fibroblasts and that this product is biologically active.

4.2 SCREENING FOR TRANSGENE

Fertilized mouse embryos were microinjected with the K14-TGF-β1 construct (Figure 1) to develop transgenic mice. Screening of these 48 mice by Southern blotting revealed that only 5 possessed the human TGF-β1 transgene (Figure 2). These five founder mice were mated to normal animals to determine stability of the transgene and the ability of these founder mice to pass the transgene on. Subsequent screening showed that only one founder mouse (Fo #33) was capable of producing progeny that tested positive for the K 14-TGF-β1 construct. Also determined by the study of the F1 generation of Fo #33 was

that the founder mouse was heterozygous for the transgene and that it was inherited in an autosomal manner.

4.3 BREEDING A TRANSGENIC MOUSE HOMOZYGOUS FOR THE K14-TGF-β1 CONSTRUCT

Mice were bred to the F2 generation to show that the transgene was stable after several generations. Because the founder mouse was heterozygous for the transgene, we thought it necessary to breed a homozygous animal that would allow us to study the differences between normal, heterozygous and homozygous animals (if any). Up to this point, animals had always been bred to a normal non-transgenic mouse. Thus any progeny testing positive for the transgene by Southern blotting had to be heterozygous. We chose only from those animals tested positive for the transgene and mated them together. Mendelian laws of inheritance dictate that by mating two heterozygous animals, 25% of the total offspring will be homozygous. Again we screened all progeny from these heterozygous mating pairs by Southern blotting and any mice positive for the transgene were tested further for homozygous transgene inheritance. We know that the transgene is not sex-linked so that if an animal is homozygous and bred with a normal animal, 100% of the offspring will be heterozygous. Thus all progeny should give a positive result from Southern blot testing (figure 3). Heterozygous animals will have 50% of the progeny testing negative for the transgene. When homozygous animals of each sex were obtained, they were mated together to keep a homozygous line of transgenic mice for future use. In total, from the development of founder mice and on, over 500 mice

were individually tested to produce homozygous and heterozygous lines of animals deriving from one founder animal (Appendix I).

4.4 REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION FOR HUMAN TGF-β1 mRNA

From the Southern blotting, we know that the transgene has been integrated into the mouse genomic DNA, but we do not know if it is properly targeted or expressed. By performing RT-PCR on extracted RNA from different organs, we can show functioning of the transgene and proper targeting by the K14 promoter. Using primers specific for the human TGF-β gene, we can differentiate exogenous and endogenous TGF-β1 expression. The results of RT-PCR show that human TGF-β1 mRNA is present only in the homozygous and heterozygous animals and is expressed only in the skin of these animals (Figure 4). To further verify that this product is from the human TGF-β1 gene and was not amplified endogenous TGF-β1, we eluted the band in question and sent it for sequencing (DNA Services Laboratory, University of Alberta) with the corresponding human or murine sense and anti-sense primers (see Materials and Methods for primer sequences). DNA sequencing confirmed the product to be from the human TGF-β1 gene and to correspond to the sequence expected to be amplified with these primers (Appendix II). To demonstrate the differences in expression between the endogenous TGF-β1 and the exogenous transgene we repeated the RT-PCR using primers specific for murine TGF-β1 (Figure 4). Mouse TGF-β1 was expressed in all the tissues tested.

4.5 PHENOTYPIC CHANGES

Expression of the K14-TGF-β1 transgene did not have any effects on fertility, weight, development or behavior. At birth, the mouse looked the same as their control counterparts. The only phenotypic difference seen was occasional scruffiness of the homozygous animals (figure 5). This scruffiness did not occur in all animals but was quite variable, at times affecting only certain animals and not their littermates. It was seen in both males and females, and initial signs of scruffiness could been seen when animals were as young as a few months to as old as one year. The only consistency was that this phenotype was not observed in the normal, or heterozygous lines (which were housed in the same area) but only in the homozygous animals. We speculate that this scruffiness is the result of the transgene targeting TGF-β1 expression to the skin of these animals. Histological sections of the skin of transgenic mice showed what appears to be a reduced dermal thickness in the homozygous animals compared to heterozygous and non-transgenic animals (figure 6A). Seen here are Masson's trichrome stained section to emphasize collagen. The homozygous skin section looks to be thinner than the others but this was not substantiated by the results of hydroxyproline analyses for collagen content (figure 6B).

4.6 SERUM TGF-β1

Serum TGF-β1 was measured in homozygous, heterozygous and normal animals (Figure 7). Levels of circulating TGF-β1 were not affected by the transgene, which further confirms the proper targeting of the K14-TGF-β1 construct. Localization of

transgene expression only to the skin of transgenic animals will minimize any multi-organ effects of the over expression of TGF-β1. The differences seen between homozygous animals (117.8 ng/mL ± 7.77 ng/mL, n=8), heterozygous animals (111.48 ng/mL ± 14.86 ng/mL, n=8) and normal animals (122.32 ng/mL ± 14.14 ng/mL, n=8) were not statistically different (ANOVA, P=0.2497).

4.7 WOUND HEALING

Four full thickness 4mm punch biopsy wounds were made in the skin on the dorsal surfaces of the mouse. The wounds were carefully examined at days 5, 7 and 9 post-wounding to observe any differences in healing. The largest contrast in healing arose at day 9 (Figure 8) when only 45% (18/40) and 37% (15/40) of the wounds made in the homozygous and heterozygous animals respectively were closed as compared to 81% (29/36) of the wounds in normal non-transgenic controls (for raw data, see Appendix IV). These results were very significant (Fisher's Exact Test, homozygous vs. normal p=0.0014; heterozygous vs. normal p=0.00014) but the differences between homozygous and heterozygous animals were not (Fisher's Exact Test, homozygous vs. heterozygous p=0.36) (Figure 11). Also significant was the difference between the homozygous and normal animals on day 7 (Fisher's Exact Test, p=0.015) but no significant difference was found between the heterozygous and normal animals (Fisher's Exact Test, p=0.18), homozygous and heterozygous (Fisher's Exact Test, p=0.18) nor in any of the day 5 comparisons (data not shown). It was seen from previous experiments that although the homozygous and heterozygous wounds re-epithelialized at a slower rate, all wounds are eventually closed by day 15 (data not shown). This experiment only followed the healing

until day 9, but previous experiments were performed that showed 100% closure of all the wounds at day 15 in the normal and transgenic animals.

4.8 TGF-β1 and α1-PROCOLLAGEN mRNA EXPRESSION BY NORTHERN ANALYSIS

Representative wounds from each animal were collected for RNA extraction and northern analysis. Each animal had two of four wounds harvested for RNA and at each time point there were ten animals for each group. One animal could only be used for one time point and so 4 different animals were needed for one group (homozygous, heterozygous, normal) to cover each time point (Day 0, 5, 7, and 9 post-wounding). One complete set for a northern blot comprised 12 animals representing all days and all three groups at different time points. Many different sets of blots were probed, and the RNA was extracted from different animals for each set.

Probing for human TGF-β1 (hTGF-β1) revealed an obvious increase in the production of hTGF-β mRNA in the homozygous animals over the heterozygous and normal animals (figure 10). This significant production of hTGF-β1 message peaked at day 5 post-wounding but dropped dramatically by day 7. A similar increase was also seen in the heterozygous animals, but to a lesser degree than in homozygous animals. Probing for hTGF-β1 in the normal non-transgenic animals gave no result as expected since the probe should be specific in differentiating exogenous human from endogenous mouse TGF-β1. Statistically significant levels of hTGF-β1 were obtained at days 0 ($p=0.0241$), 5 ($p=0.0089$) and 9 ($p=0.0269$) by ANOVA, with Bonferroni post-testing performed to

reveal that only the homozygous sets were significantly different from the normal sets at those days ($p<0.05$).

Northern analysis for mTGF-β1 does not demonstrate any great contrast between the transgenic animals and the normal controls (Figure 11). The peak expression appears to occur at day 5 post wounding as well.

Probing for mRNA for the pro-α1 chain of type I collagen also revealed differences in its expression between the different groups of animals (Figure 12). Once again the homozygous animals appeared to be expressing the most mRNA compared to the other groups, but this time the peak was at day 9. Although the heterozygous animals expressed less message for this protein than did the homozygous animals, they expressed more message than the normal animals.

Collagen type III mRNA was also looked at but no differences were seen except in one particular set of blots. From the autoradiogram, one homozygous animal showed a peak collagen type III message at day 7 as opposed to day 9 as seen in the heterozygous and only a slight increase in message in the normal animals (Figure 13). This result was unique to this one set of blots and was not seen in any other sets, but the earlier induction of collagen type III expression may be significant even though a strong signal was not seen in all sets.

Several sets of autoradiograms were scanned into the NIH Imaging program for quantitative densitometry. Each sample was corrected for loading by 18S rRNA probing of the blots. In most samples, day 0 post-wounding values in the normal animals were taken as the reference point and assigned an arbitrary value of '1'. All other samples were

compared to this value and plotted accordingly (Appendix V). The results from this attempt to quantitate the autoradiograms were so variable that we could not substantially conclude anything.

4. 9 HYDROXYPROLINE CONTENT OF SKIN AND WOUND SAMPLES

Skin samples were collected from normal, heterozygous and homozygous animals with a 4mm punch biopsy and analyzed colorimetrically for hydroxyproline as a measurement of collagen content. There were no significant differences between the normal (102.7 $\pm$ 4.6μg, n=5), heterozygous (123.0 $\pm$ 62.3μg, n=5) and homozygous (133.0 $\pm$ 47.0μg, n=5) groups in hydroxyproline content (Figure 6B and 14). Large variations in hydroxyproline content were not due to the size of the biopsy (Appendix VI, VII). Therefore the apparently thinner dermis seen in histological sections was not confirmed.

Wounds were collected at days 5, 7 and 9 post wounding with a 6 mm punch biopsy and analyzed for hydroxyproline content using the colorimetric method. A summary of the data for all three groups over the entire wound healing time course can be seen in Figure 14. Significantly different hydroxyproline levels were obtained at days 5 and 9. Day 5 samples showed biopsies from homozygotes (106.1 $\pm$ 32.4μg, n=5) and heterozygotes (92.3 $\pm$ 14.8μg, n=5) were significantly higher in hydroxyproline content (P=0.0170, ANOVA) compared to those from the normal control animal biopsies (61.854 $\pm$ 7.0934μg, n=5). At day 9, biopsies from homozygous (128.4 $\pm$ 29.3μg, n=5) and normal animals (119.4 $\pm$ 24.8μg, n=5) showed higher hydroxyproline levels than biopsies collected from heterozygous animals (89.5 $\pm$ 8.7μg, n=5); but these values are just only

borderline significant (P=0.0465, ANOVA) and may not represent a true biological difference. Biopsies collected on day 7 post wounding were not significantly different in hydroxyproline content (P=0.3022, ANOVA) between the homozygous (133.30 ± 48.359μg, n=5), heterozygous (169.31 ± 22.669μg, n=5) and normal (138.26 ± 38.246μg, n=5) biopsy samples. Differences in hydroxyproline content are not due to variations in biopsy size because the wet weight, dry weight and percentage of water in the biopsies are all relatively similar (Appendices VI, VII, VIII).

4.10 HISTOLOGY and IMMUNOHISTOCHEMISTRY

Tissues for histological sections were collected from wounds harvested at days 5, 7 and 9 post-wounding to observe any morphological changes and differences in TGF-β1 in the skin. On day 5 post-wounding (Figure 15), all wounds were still open and evidence of the fibrin clot is seen. The advancing epithelium moving underneath the scab is also demonstrated. Interestingly, the staining for TGF-β1 is restricted in the normal samples to the migrating epithelium. In the homozygous and heterozygous animals, more intense staining is seen and staining is also seen in the epithelium distal from the wound. This staining pattern is seen at all days which shows TGF-β1 across the epithelium in homozygous and heterozygous animals. Interestingly, the source of the TGF-β1 in the transgenic mice appears to be the basal layers of the epithelium, as opposed to the suprabasal keratinocytes in the normal animals (Figures 15, 16, 17).

4.11 TGF-β1 in the SERUM

Serum was collected from euthanised animals at each time point for each group (Figure 18). Mean circulating TGF-β1 levels in the serum for the homozygous animals throughout the different days post-wounding were 117.9 ± 7.8 ng/mL (n=8) at day 0, 108.7 ± 14.7 ng/mL (n=9) at day 5, 121.7 ± 9.3 ng/mL (n=6) at day 7 and 120.4 ± 8.8 ng/mL (n=5) at day 9. For the heterozygous animals, the levels were 111.5 ± 14.9 ng/mL (n=8), 113.5 ± 12.3 ng/mL (n=8), 109.1 ± 13.9 ng/mL (n=6) and 104.5 ± 3.9 ng/mL (n=5) for days 0, 5, 7 and 9 post-wounding respectively. Normal animals produced levels of circulating TGF-β1 of 122.3 ± 14.1 ng/mL (n=8), 117.0 ± 12.8 ng/mL (n=8), 112.8 ± 11.7 ng/mL (n=6) and 108.8 ± 9.4 ng/mL (n=5) from days 0 to 9 post-wounding. There were no statistical differences in the levels of circulating TGF-β1 when comparing the different groups at day 0 (ANOVA, P=0.2497), day 5 (ANOVA, P=0.4528), and day 7 (ANOVA, P=0.1970). On day 9, the differences in serum TGF-β1 between the three groups appear significant (ANOVA, P=0.0193), but that is probably due to the uncharacteristically small standard deviation for the heterozygous animal samples on that day (±3.90 ng/mL). Also considered were possible changes in circulating levels of TGF-β1 within each group over the time course of wound healing. Once again, no significant differences were seen between results for the homozygous animals (ANOVA, P=0.1140), heterozygous animals (ANOVA, P=0.6416) or normal non-transgenic animals (ANOVA, P=0.2707). Thus although northern analysis shows a large increase in human TGF-β1 mRNA at day 5 for the homozygous and heterozygous animals, this effect is localized to the wound site and does not produce increases in serum TGF-β1.

4.12 PAI/L ASSAY FOR *IN VIVO* TGF-β1

The PAI/L assay was used to determine the amount of active and total TGF-β1 present in the wounds at the different time points post-injury (Figure 19A, B). Both the active and total levels of TGF-β1 rise during the healing time course, peaking between days 5 and 7. The level of total TGF-β1 in the normal animals at day 9 (247.3 $\pm$ 54.1 pg) declined to those similar to the day 0 samples (306.8 $\pm$ 75.4 pg). However, in the transgenic animals the total TGF-β1 remained high. At day 9 post-wounding, the amount of TGF-β1 present at this time point was significantly different between the three groups of animals ($P<0.001$, ANOVA, $n=3$). Post-tests to calculate Bonferroni p value revealed no differences in the levels between the homozygous (1121.0 $\pm$ 82.1 pg) and heterozygous animals (779.5 $\pm$ 228.1 pg), but significantly increased TGF-β1 in both the homozygous animals and heterozygous animals over the normal animals (HM vs. N, $p<0.01$; HT vs. N, $p<0.05$). Due to the large inter-animal variability and limited numbers of animals, other time points were not statistically significant although the data does suggest increased total TGF-β1 present in the wounds of transgenic animals resulting in higher levels of active TGF-β1 as well.

CHAPTER FIVE

DISCUSSION

48 live births resulted from the implantation of embryos microinjected with the K14-hTGF-β1 transgene. Southern analysis revealed that five of these forty-eight animals had the transgene integrated into their DNA. This number is not unreasonable as only 10-40% of the mice generated from this procedure is expected to have transgene integration (Ho et al, 1994). Further breeding of these five founder mice showed only one (Fo#33) was capable of passing the transgene onto its progeny. These matings also revealed that the transgene integrated in a heterozygous fashion and had integrated into an autosomal chromosome (based on the pattern of transgene inheritance). It was this founder that was used to breed subsequent homozygous animals. We were able to produce a line of viable homozygous animals from the breeding of the heterozygous progeny of Fo#33. To ensure that the animals bred were indeed homozygous each was mated to a normal mouse. If an animal was homozygous, we could detect the transgene in all the resulting progeny by Southern blotting. If any progeny gave a negative result from the Southern blotting, the parent animals in question would immediately be excluded as homozygous.

There was some initial concern that the over-expression of TGF-β in the homozygous animals would produce some developmental problems or even excessive fibrosis resulting in death (as seen in the albumin-promoted TGF-β transgenic animals (Sanderson et al, 1995; Kopp et al, 1996) or keratin-1-promoted TGF-β transgenic animals (Sellheyer et al, 1993)). However, we expected that our transgene could be incorporated into a viable line of homozygous animals. First, we ligated our transgene

with a keratin 14 promoter which should target expression specifically to the skin. The most important difference from previous attempts was our use of the complete cDNA sequence of the human TGF-β1 gene which included the unmodified LAP to produce latent TGF-β1. With the albumin promoter, circulating levels of active TGF-β would be increased thus explaining the multi-organ fibrosis observed which eventually resulted in the death of the animals. The cysteines at position 223 and 225 of the LAP were modified to serines which produced constitutively active TGF-β in the transgenic mice in other studies (Brunner et al, 1989; Cui et al, 1995; Kopp et al, 1996; Samuel et al, 1993; Sanderson et al, 1995; Sellheyer et al, 1993). In addition to studying the effects of TGF-β in the skin, our animals may be useful in the study of mechanisms of activation TGF-β. Use of the latent TGF-β transgene allows production of transgenic mice that will behave very similarly to normal mice until stimulated (for example by wounding). The response of the transgenic animals will only differ in the amount of TGF-β being produced in the epidermis and be more similar to a normal physiological response to TGF-β by requiring activation of the molecule.

Proper functioning of the transgene must be demonstrated. Initial attempts using northern analysis for the detection of RNA were unsuccessful and therefore we decided to use RT-PCR. Extracted RNA from different organs from all three lines (homozygous, heterozygous and normal animals) was treated with DNAse to prevent any amplification of DNA left over from the extraction. The cDNA used for PCR was made using specific human TGF-β1 primers (see Material and Methods for primer sequences). Only the homozygous and heterozygous animals showed a product and even then, it was demonstrated only in the skin and not in any other organs. We have shown that the

transgene is being expressed in the mice and that it is properly targeted to the skin of these transgenic animals using the keratin 14 promoter as successfully as in previous studies using the K1 (Sellheyer et al, 1993) or the K10 (Cui et al, 1995) promoters. For comparison, mouse TGF-β mRNA was also looked at by this method and the results show that mouse TGF-β is expressed endogenously in all tissues. To ensure that the PCR products were specific, the bands were electroeluted out of the gel and the DNA precipitated prior to being sent for sequencing. Sequencing results confirmed that the products of the expected base pair length were human or mouse TGF-β1.

Although we had not expected any obvious phenotypic differences between the transgenic mice and the normal animals prior to wounding, we did observe some scruffiness in the homozygous line. It appeared that at varying ages (between 1 month and one year, data not shown) the homozygous mice would show signs of inhibited hair growth that resulted in a scruffy appearance in these animals. TGF-β1 is a known negative regulator of epithelial cell growth (Gleizes et al, 1997; Sachs, 1996; Sporn et al, 1986). The cells in the hair follicle are continually dividing and active and TGF-β1 has been shown to be able to stimulate cells in the resting state yet inhibit the same cell types when they are active (Wahl, 1994). Not only is TGF-β1 constitutively expressed in the hair follicles (Sullivan et al, 1995), keratin 14 is synthesized in the basal layer of the outer root sheath (Vassar et al, 1989) and therefore the appearance of transgenic animals with obvious changes in hair growth is not unexpected. The fact that only the homozygous animals show this phenotype suggests that higher levels of TGF-β in these animals and the delayed onset of this phenotype may involve activation of TGF-β1.

In our transgenic animals there were no measurable increases in circulating TGF-β1 due to the transgene. Previous transgenic animals have been shown to have increased circulating TGF-β1 that resulted in multi-organ effects such as fibrosis and lesions which resulted in the early death of most animals (Sanderson et al, 1995). Once again, differences in the transgene composition can explain the lack of differences in the serum levels. Targeting of the transgene to the epidermis may limit the amount of exogenous TGF-β1 entering the circulation despite the fact that *in vitro* studies have shown the possibility of keratinocyte products passing through the basement membrane into the dermis (Kondo et al, 1997; Katz et al, 1994). The lack of increased levels of TGF-β1 in the circulation indicates a low risk of multi-organ fibrosis and systemic problems relating to increased TGF-β1 synthesis which would compromise the survival of the animals.

Histologically, we did not observe any dramatic effect of the transgene upon the structure of the skin. Initially in some sections the dermis appeared to be thinner in the homozygous animals compared to the control or heterozygous animals, but this observation could not be substantiated when the collagen content was measured by hydroxyproline analysis. If the TGF-β1 synthesized from the transgene were capable of passing through the basement membrane, we would expect an increase in collagen production in the dermis. The resulting lack of change in collagen production in the homozygous and heterozygous animals could be attributed to the lack of sufficient activation of TGF-β1 from the transgene. Without the cleavage of the LAP from the mature protein, the total amount of TGF-β1 present does not necessarily correspond to biologically active TGF-β1 (Theodoresu et al, 1991).

Previous wound healing experiments with TGF-β1 involved the introduction of active TGF-β1 into the dermis through injections (Roberts et al, 1986; Sporn et al, 1983), cytomegalovirus transfection of granular tissue (Benn et al, 1996) or even the surgical implantation of cellulose disks under grafted tissue to slowly release the cytokine over several days (Lin et al, 1995). Using full thickness biopsy wounds, we were able to observe that most of wounds healed mainly by re-epithelialization, not contraction, and that the transgene had significant effects on the rate of closure of the wounds. TGF-β1 appears to reduce the rate of healing by re-epithelization which is not unexpected due to its effects seen *in vitro* on the inhibition of cell proliferation (Kim et al, 1989;Sporn et al, 1986). The rate of healing was significantly reduced in the homozygous and heterozygous animals by day 9-post-wounding.

Northern analysis for human TGF-β mRNA revealed maximal production at day 5 post-wounding and this increase translates into increased protein synthesis of the latent TGF-β1 protein. Plasmin has been demonstrated to be capable of activating latent TGF-β (Lyons et al, 1988; 1990). In normal wound healing, the clot is being broken down by plasmin by day 5 post wounding and this is a probable mechanism for the activation of TGF-β1 *in vivo*. Activation of latent TGF-β1 by plasmin at day 5 in the transgenic animals could lead to reduced proliferation of keratinocytes and therefore reduced re-epithelialization. In addition to the epidermis, full thickness biopsies remove the dermis, reducing the underlying collagen that may play a role in the activation of TGF-β (Gleizes et al, 1997). The hydroxyproline analysis for collagen production is significantly increased at days 5 (peak TGF-β mRNA production) and 9. Although this result does not correlate with the northern analysis, it is probably due to large variations between animal

samples in each group even after RNA loading was corrected using 18S rRNA. Although the northern analysis reveals increased mRNA for the pro-α1(I) chains of type I collagen in some sets of blots by day 9, this phenomenon was not reflected to the same extent in the hydroxyproline analyses.

Immunohistochemistry confirms the northern analysis for TGF-β1 mRNA, showing greater staining for TGF-β1 in sections from transgenic animals over the normal animals at day 5. Interestingly, staining for TGF-β1 is greatest in the advancing keratinocytes in the normal animals, but in the transgenic animals, there appears to be increased TGF-β1 across the entire epidermis in all days post-wounding. Staining is also in the basal layer of the epidermis in the transgenic animals (also demonstrated by Vassar et al (1989)) as opposed to the suprabasal layer for the normal which could be result of the difference between K14-driven expression and endogenous TGF-β. Over the time course of wound healing, basal staining of TGF-β1 can be seen and histological sections of the wounds over time confirm that re-epithelialization is faster in the normal animals. By day 9, the wound has fully re-epithelialized and shows keratinization. The more intensely stained regions of the specimens from the homozygous and heterozygous animals represents increased TGF-β1 production and thus the retardation of re-epithelization in these animals can be ascribed to the anti-proliferative effects of TGF-β on epidermal cells (Kim et al, 1989; Sporn et al, 1986).

Serum TGF-β1 levels reveal that the effects of the transgene even after wounding are still localized to the epithelium of the animals. It is suggested that the biopsy wounds are insufficient to produce a systemic effect of the transgene. Only four full-thickness 4 mm biopsy wounds were made on the dorsal surface of these animals which represents a

small fraction of the total body surface area. Other types of wounding will be examined in future studies. An example of work in progress includes laser-induced thermal injuries in which attempts will be made to produce a partial thickness wound. Partial thickness thermal injuries will damage the epithelium, induce wound healing but leave areas of the underlying dermis intact that may be necessary for activation of TGF-β.

The PAI/L assay allowed us to quantitate the *in vivo* amount of TGF-β1 present in the wounds. At all the time points, the transgenic animals appeared to possess higher levels of both active and total TGF-β1. Peak levels of total TGF-β1 occurred around day 5 post-wounding which corresponds to the maximum production of TGF-β1 mRNA seen in the northern analysis. Generally the active TGF-β1 profile mimicked the amount of total TGF-β1 present but the highest amounts of active TGF-β1 occur at day 7 post-wounding as opposed to day 5 for the total TGF-β1 present. Our use of a latent TGF-β1 transgene product could explain this delay between the amount of total TGF-β1 being produced and the amount of active TGF-β1 being detected. Activation *in vivo* is not fully understood, but plasmin is suggested to be a major player. It is present to breakdown the fibrin clot during healing and had been shown to be able to activate TGF-β1 *in vitro* (Lyons et al, 1988; 1990). Interestingly, the highest levels of active TGF-β1 occur at day 7 post-wounding which is the same time as when differences in wound re-epithelialization is observed. The increased TGF-β1 present appears to delay the keratinocyte re-epithelialization of the open wounds. Overall, the amount of active TGF-β1 is approximately 5% of the total present for all three groups of animals. Activation mechanisms may have been hindered by the use of full thickness biopsies as ECM has been shown to maybe play a role in TGF-β1 activation (Gleizes et al, 1997; Taipale et al,

1996). Another reason may be the activation of TGF-β1 by plasmin is only secondary to its role in the breakdown of its natural substrate fibrin and therefore may limit its availability.

TGF-β1 is a potent cytokine that is found in most tissues (Assoian et al, 1983; Roberts et al 1993) and whose role in vivo involves wound healing (Benn et al, 1996; Mustoe et al, 1987; Roberts et al, 1986;1993; Sporn et al, 1983), immune modulation (Assoian et al, 1983, Espevik et al, 1990; Li et al, 1993), extracellular matrix production (Gleizes et al, 1997; Zhang et al, 1995) and control of cell proliferation (Gleizes et al, 1997; Glick et al, 1993; Kim et al, 1989; Sachs et al, 1996). Based on previous studies, we expected the increase in TGF-β1 to have an effect on the collagen synthesis. Studies by Davidson et al (1993) used exogenously added TGF-β1 to show an increase in both elastin and collagen synthesis. But in our study, the highest amount of active TGF-β1 achieved was 85.3 $\pm$ 39.4 pg/animal (homozygous animals, day 7). Davidson tested different amounts of TGF-β1 and found the optimal production of collagen occurred was when 10 ng/mL of TGF-β1was added. Therefore, the levels of TGF-β1 required for an extremely significant increase in the production of collagen may require higher levels than physiologically present in this study.

Deitch *et al* (1983) showed that the most important factor in determining wound healing problems such as hypertrophic scarring was the length of time it took the wounds to heal. One third of the wounds that took between 10-14 days to heal would develop hypertrophic scarring and if the wounds took longer than 21 days to heal, the rate would increase to 78%. Other factors that proved significant in the study included inflammation and wound tension. In our study, wounds healed by day 15 in all three groups of animals.

There were no obvious signs of infection or excessive inflammation as surgical procedures were performed under strict sterile conditions. Wound tension was also negligible, as mice are loosed skinned animals that have very little skin tension. The delay in re-epithelialization is a significant factor in the development of hypertrophic scars shown in human studies, but alone may not be sufficient in producing exaggerated ECM formation during wound healing in mice.

CHAPTER SIX

CONCLUSIONS AND FUTURE EXPERIMENTS

6.1 CONCLUSIONS

It was believed and proven that the detrimental effects of excessive TGF-β1 in the mice could be overcome by using a transgene that encoded the latent form of the cytokine. This enabled us to produce not only heterozygous animals, but a homozygous line that expressed the transgene specifically in the skin transgenic animals. This expression is limited to the epidermis, as demonstrated by the lack of change in collagen production in the dermis (based on hydroxyproline content) and circulating TGF-β1 levels. The only phenotypic difference caused by the transgene is the appearance of "scruffiness" in some of the homozygous animals.

Healing of full thickness punch biopsies showed a reduced rate of re-epithelialization in the transgenic animals. This result is contrary to many wound studies that have shown improvement in healing from the addition of exogenous TGF-β1. However, such studies used incisional wounds and the application of recombinant active TGF-β1 into the dermis to produce results on collagen production and tensile strength. Our wound healing study differs in several ways which includes the types of wounds studied, source of TGF-β and the use of excessive latent TGF-β.

The major impediment to the action of latent TGF-β1 is the requirement for activation. Measurements of both total and activated TGF-β1 *in vivo* reveal that only approximately 5% of the total TGF-β1 becomes activated in our wound healing model.

Were this large fraction of latent TGF-β1 activated, it could potentially produce a much stronger dermal response during wound healing.

This thesis has shown that the K14-TGF-β1 transgenic mouse is viable using a latent form of the gene. The transgene is being properly expressed only in the skin of these animals. Upon wounding, the excessive TGF-β1 has a detrimental effect upon re-epithelialization. The transgene is also reported to increase the hydroxyproline content of skin during wound healing, which further confirms the role of TGF-β1 as a fibrogenic cytokine. Although we were not able to produce a hypertrophic scar in the animals, we were able to see effects from excessive TGF-β1 that may play a role in the pathogenesis of this fibroproliferative disorder.

6.2 FUTURE APPLICATIONS

Regrettably the attempts to culture keratinocytes from the transgenic animals have been unsuccessful. This may be due to the over-expression of TGF-β1 from these cells which would therefore require the addition of neutralizing antibodies to the culture medium.

We have not been able to detect any increase in TGF-β1 protein level in the serum of the transgenic animals, otherwise they may be useful in the study of systemic inflammatory diseases (as reviewed by Bhan et al, 1999) through the mating of different transgenic animals. Luckily, there has been some success in the development of transgenic mouse models for inflammatory skin diseases. Carroll et al (1995) reported phenotypic changes in mice occurred to resemble those of psoriasis from the expression of specific integrin combinations in the suprabasal epidermal layers. Using the involucrin

promoter to specifically target integrin beta 1 alone or in combination with alpha 2 or 5 integrins, these mice exhibited epidermal hyperproliferation and skin inflammation. It would be interesting to investigate whether these phenotypic changes could be reduced by excessive TGF-β1 through the cross mating between this transgenic mouse and our K14-TGF-β1 mouse. The study of skin inflammation could also be performed without breeding of two transgenic mouse lines. Simply by the introduction of naked IL-6 DNA to the skin of rats, Sawamura et al (1998) was able to produce changes related to those seen in inflammatory skin diseases. If such a procedure were performed in the K14-TGF-β1 transgenic mouse, we may be able to observe the role of TGF-β1 as an anti-inflammatory agent *in vivo*. In addition, one of the concerns in our wound healing study was the lack of inflammation seen which could contribute to the low levels of activated TGF-β1.

We are most interested in the role of TGF-β1 in wound healing. In our experiments, we were able to detect a large fraction of the total TGF-β1 being produced that was not activated. The exact mechanism of the activation of TGF-β1 *in vivo* is still relatively unknown. The amount of activated TGF-β1 we measure *in vivo* may be insufficient to produce dramatic dermal response. Other groups have been successful in administering excessive TGF-β1 during wound healing by injection or the implantation of polyvinyl alcohol sponges. We could use these delivery techniques to introduce activating factors, for example plasmin, that would allow us to study the full effects of TGF-β1 in wound healing.

In addition to wound healing, this transgenic mouse may be useful to study the activation of TGF-β1 *in vivo*. Through topical application or subcutaneous injections of

different agents we may provide some insight as to which factors are important attempt in the activation of TGF-β1 *in vivo*. Studies *in vitro* have reported the inhibition of TGF-β1 activation by the addition of excess M-6-P and antibodies to plasmin.

Perhaps a different wound may produce the changes in the dermis we had initially hoped to observe. Currently, we are investigating partial thickness laser-induced thermal injuries that would damage the epithelium but leave some of the underlying dermis intact that may be necessary for activation. The laser wounds will increase the chances of a strong inflammatory response, as injured tissue is present and not removed during healing.

LITERATURE CITED

Amento, E.P.E, and Beck, L.S. TGF-β and wound healing. CIBA Foundation Symposium 1991: 115-29.

Anonymous. Endocrine, metabolism and immunologic functions of keratinocytes. Ann. NY Acad. Sci. 1988; 548: 262-70.

Anzano, M.A., Roberts, A.B, and Sporn, M.B. Anchrorage-independent growth of primary rat embryo cells is induced by platelet-derived growth factor and inhibited by type-beta transforming growth factor. J. Cell. Physiol. 1986; 126: 312-8

Ashcroft, G.S., Dodsworth, J., Van Boxtel, E., Tarnuzzer, R.W., Horan, M.A., Schultz, G.S. and Ferguson, M.W. Estrogen accelerates cutaneous wound healing associated with an increase in TGF-β1 levels. Nature Medicine 1997; 3(11): 1209-15.

Ashwell, G and Harford, J. Carbohydrate-specific receptors of the liver. Annual Review of Biochemistry 1982; 51: 531-54.

Assoian, R.K., Meyers, C.A., Miller, D.M., and Sporn, M.B. Transforming growth factor-beta in human platelets. Identification of a major storage site, purification and characterization. Journal of Biological Chemistry 1983; 258(11): 7155-60.

Barral, A., Teixeira, M., Reis, P., Vinhas, V., Costa, J., Lessa, H., Bittencourt, A.L., Reed, S., Carvalho, E.M., and Barral-Netto, M. Transforming growth factor-beta in human cutaneous leishmaniasis. American Journal of Pathology 1995; 147(4): 947-54.

Beck, L.S., DeGuzman, L., Lee, W.P., Xu, Y., Siegel, M.W., and Amentp, E.P. One systemis administration of transforming growth factor-β1 reverses age- or glucocorticoid-impaired wound healing. J. Clin. Invest. 1993; 92: 2841-49.

Benn, S.I., Whitsitt, J.S., Broadley, K.N., Nanney, L.B., Perkins, D., He, L., Patel, M., Morgan, J.R., Swain, W.F., Davidson, J.M. Particle-mediated gene transfer with transforming growth factor-β1 cDNAs enhances wound repair in rat skin. J. Clin. Invest. 1996; 98(12): 2894-2902.

Bhan, A.K., Mizoguchi, E., Smith, R.N., and Mizoguchi, A. Colitis in transgenic and knockout animals as models of human inflammatory bowel diseases. Immuno. Rev. 1999; 169:195-207.

Birkedal-Hansen, H., Werb, Z., and Welgus, H.G. Matrix metalloproteinases and inhibitors. Stuttgart, Ger: Gustan, Fisher, Verlag, 1992.

Bloom, B.B., Humphries, D.E., Kuang, P.P, Fine, A., and Goldstein, R.H. Structure and expression of the promoter for the R4/Alk5 human type I transforming growth factor-beta receptor: regulation by TGF-β. Biochemica et Biophysica Acta. 1996; 1312(3): 243-8.

Border, W.A., and Ruoslahti, E. Transforming growth factor-beta in disease: the dark side of tissue repair. J Clin. Invest. 1992; 90(1): 1-7.

Border, W.A., Noble, N.A., Yamamoto, T., Harper, J.R., Yamaguchi, Y., Pierschbacher, M.D., and Ruoslahti, E. Natural inhibitor of transforming growth factor-β protects against scarring in experimental kidney disease. Nature 1992; 360: 361-4.

Brettell, L.M. and McGowan, S.E. Basic fibroblast growth factor decreases elastin producion by neonatal rat lung fibroblasts. Am. J., Respir. Cell Mol. Biol. 1994; 10: 306-15.

Bronzert, D.A., Bates, S.E., Sheridan, J.P., Lindsey, R., Valverius, E.M., Stampfer, M.R., Lippman, M.E., and Dickson, R.B. Transforming growth induces platelet-derived growth factor (PDGF) messenger RNA and PDGF secretion while inhibiting growth in normal human mammary epithelial cells. Molecular Endocrinology 1990; 4(7): 981-9.

Brouty-Boye, D. & Zetter, B.R. Inhibition of cell motility by interferon. Science 1980; 208: 516-8.

Brunner, A.M., Marquardt, H., Malacko, A.R., Lioubin, M.N., and Purchio, A.F. Site-directed mutagenesis of cysteine residues in the pro-region of the transforming growth factor-beta 1 precursor. Expression and characterization of mutant proteins. J. Biol. Chem. 1989; 264(23): 13660-4.

Carroll, J.M., Romero, M.R., and Watt, F.M. Suprabasal integrin expression in the epidermis of transgenic mice results in developmental defects and a phenotype resembling psoriasis. Cell 1995; 83(6): 957-68.

Cebon, J., Nicola, N., Ward, M., Gardner, I., Dempsey, P., Layton, J., Duhrsen, U., Burgess, A.W., Nice, E., and Morstyn, G. Granulocyte-macrophage colony stimulating factor from human lymphocytes-the effect of glycosylation on receptor binding and biological activity. J. Biol. Chem. 1990; 265: 4483-91.

Chambaz, E.M., Souchelnitskiy, S., Pellerin, S., Defaye, G., Cochet, C., and Feige, J-J. Transforming growth factor-βs: a multifunctional cytokine family. Horm. Res. 1996; 45: 222-6.

Chen, F. & Weinberg, R.A. Biochemical evidence for the autophosphorylation and transphosphorylation of TGF-β receptor kinases. Proc. Natl. Acad. Sci. USA 1995; 92:1565-69.

Childs, C.B., Proper, J.A., Tucker, R.F., and Moses, H.L. Serum contains a platelet-derived transforming growth factor. Proc. Natl. Acad. Sci. USA 1982; 79(17): 5312-6.

Chomczynski, P. and Sacchi, N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Analytical Biochemistry 1987; 162: 156-9.

Clymer, M.A., Schwaber, M.K., and Davidson, J.M. The effects of keratinocyte growth factor on healing of tympanic membrane perforations. Laryngoscope 1996; 106:280-5.

Coleman, C., Tuan, T-L., Buckley, S., Anderson, K.D., and Warburton, D. Contractility, transforming growth factor-β, and plasmin in fetal skin fibroblasts: role in scarless wound healing. Pediatric Research 1998; 43(3): 403-9.

Connor, T.B., Roberts, A.B., Sporn, M.B., Danielpour, D., Dart, L.L., Michels, R.G., DeBustros, S., Enger, C., Kato, H. and Lansing, M. Correlation of fibrosis and transforming growth factor-beta type 2 levels in the eye. Journal of Clinical Investigation 1989; 83(5): 1661-6.

Cui, W. Fowlis, D.J., Cousins, F.M., Duffie, E., Bryson, S., Balmain, A., and Akhurst, R.J. Concerted action of TGF-β1 and its type II receptor in control of epidermal homeostasis in transgenic mice. Genes & Development 1995; 9: 945-55.

Davidson, J.M., Zoia, O., and Liu, J-M. Modulation of transforming growth factor-beta 1 stimulated elastin and collagen production and proliferation in porcine vascular smooth muscle cells and skin fibroblasts by basic fibroblast growth factor-α, and insulin-like growth factor-1. J. Cell. Phys. 1993; 155:149-56.

De Larco, J.E. and Todaro, G.J. Growth factors from murine sarcoma virus-transformed cells. Proc. Natl. Acad. Sci. USA 1978; 75(8): 4001-5.

Deitch, E.A., Wheelahan, T.M., Rose, M.P., Clothier, J., and Cotter, J. Hypertrophic burn scars: analysis of variables. The Journal of Trauma 1983; 23(10): 895-8.

Dennis, P. and Rifkin, D.B. Cellular activation of latent transforming growth factor beta requires binding to the cation-independent mannose-6-phosphate /insulin-like growth factor II receptor. Proc. Natl. Acad. Sci. USA 1991; 88:580-4.

Desmouliere, A., Geinoz, F., Gabbiani, F., and Gabbiani, G. Transforming growth factor-beta-1 induces alpha-smooth muscle actin expression in granulation tissue myofibroblasts and in quiescent and growing cultures fibroblasts. J. Cell Biol. 1993; 122: 103-111.

Detmar, M., Brown, L.F., Schon, M.P., Elicker, B.M., Velasco, P., Richard, L., Fukumura, D., Monsky, W., Claffey, K.P., and Jain, R.K. Increased microvascular density and enhanced leukocyte rolling and adhesion in the skin of VEGF transgenic mice. J. Invest. Derm. 1998; 111(1): 1-6.

Diebold, R.J., Eis, M.J., Yin, M, Ormsby, I., Boivin, G.P., Darrow, B.J., Saffitz, J.E., and Doetschman, T. Early-onset multifocal inflammation in the transforming growth factor-beta 1 null mouse is lymphocyte mediated. Proc. Natl. Acad. Sci. USA 1995; 92(26): 12215-9.

Ebner, R., Chen, R.H., Shum, L., Lawler, S., Zioncheck, T.F., Lee, A., Lopez, A.R., and Derynck, R. Cloning of a type I TGF-β receptor and its effect on TGF-β binding to the type II receptor. Science 1993; 260: 1344-8.

Eckert, R.L. Structure function and differentiation of keratinocytes. Phys. Rev. 1989; 69: 1316-46.

Elias, J.A., Geba, G.P., Tang, W., Schilz, R., Zheng, T., DiCosmo, B., and Flavell, R.A. Transgenic modeling of cytokines in the investigation of pulmonary disease. Chest 1996; 109(suppl): 69S-75S.

Espevik, T., Waage, A., Faxvaag, A., and Shalaby, M.R. Regulation of interleukin-2 and interleukin-6 production from T-cells: involvement if interleukin-1β and transforming growth factor-β. Cell. Immunol. 1990; 126: 47-56.

Feldman, S.R., Rosenberg, M.R., Ney, A., Michalopoulos, G., and Pizzo, S.V. Binding of alpha$_2$macroglobulin to hepatocytes: mechanism of in vivo clearance. Biochem. Biophys. Res. Commun. 1985; 128: 795-802.

Finkelman, R.D., Linkhart, T.A., Mohan, S., Lau, K.H., Baylink, D.J., and Belle, N.H. Vitamin D deficiency causes a selective reduction in deposition of transforming growth factor-beta in rat bone: possible mechanism for impaired osteoinduction. Proc. Natl. Acad. Sci. 1991; 88: 3657-60.

Flaumenhaft, R., Abe, M., Mignatti, P., and Rifkin, D.B. Basic fibroblast growth factor-induced activation of latent transforming growth factor beta in endothelial cells: regulation of plasminogen activator activity. J. Cell Biol. 1992; 118(4): 901-9.

Flaumenhaft, R., Abe, M., Sato, Y. et al. Role of the latent TGF-beta binding protein in the activation of latent TGF-beta by co-cultures of enothelial and smooth muscle cells. J. Cell. Biol. 1993; 120: 995-1002.

Flaumenhaft, R., Kojima, S., Abe, M., and Rifkin, D.B. Activation of latent transforming growth factor-beta. Advances in Pharmacology 1993; 24:51-76.

Gabrielian, K., Osusky, R., Sippy, B.D., Ryan, S.J., and Hinton, D.R. Effect of TGF-β on interferon-γ induced HLA-DR expression in human retinal pigment epithelial cells. Investigative Opthalmology & Visual Science 1994; 35(13): 4253-9.

Gamble, J.R. & Vadas, M.A. Endothelial adhesiveness for blood neutrophils is inhibited by transforming growth factor-beta. Science 1988; 242: 97-9.

Gamble, J.R. & Vadas, M.A. Endothelial cell adhesiveness for human T lymphocytes is inhibited by transforming growth factor-beta 1. Am. J. Pathol. 1979; 146(4): 1149-54.

Gamble, J.R., Khew-Goodall, Y., and Vadas, M.A. Transforming growth factor-β inhibits E-selectin expression on human endothelial cells. J Immunol. 1993; 150: 4494-503.

Ghahary, A., Pannu, R., and Tredget, E.E. Fibrogenic and anti-fibrogenic factors in wound repair. Advances in Structural Biology. 1996; 4: 197-231.

Ghahary, A., Tredget, E.E., Chang, L.-J., Scott, P.G. and Shen, Q. Genetically modified dermal keratinocytes express high levels of transforming growth factor-β1. The Journal of Investigative Dermatology 1998; 110(5): 800-5.

Gleizes, P-E., Mungler, J.S., Nunes, I., Harpel, J.G., Mazzieri, R., Noguera, I., and Rifkin, D.B. TGF-β latency: biological significance and mechanism of activation. Stem Cells 1997; 15: 190-7.

Glick, A.B., Kulkarni, A.B., Tennenbaum, T., Hennings, H., Flanders, K.C., O'Reilly, M., Sporn, M.B., Karlsson, S., and Yuspa, S.H. Loss of expression of transforming growth factor-beta in skin and skin tumors is associated with hyperproliferation and a high risk for malignant conversion. Proc. Natl. Acad. Sci. USA 1993; 90: 6076-80.

Gollnick, S.O., Cheng, H.L., Grande, C.C., Thompson, D., and Tomasi, T.B. Effects of transforming growth factor-beta on bone marrow macrophage Ia expression induced by cytokines. Journal of Interferon & Cytokine Research 1995; 15(5): 485-91.

Gordon, J.W., Scangos, G.A., Plotkin, D.J., Barbosa, J.A., and Ruddle, F.H. Genetic transformation of mouse embryos by microinjection of purified DNA. Proc. Natl. Acad. Sci. USA 1980; 77(12): 7380-4.

Grainger, D.J., Kemp, P.R., Liu, A.C., Lawn, R.M., and Metcalfe, J.C. Activation of transforming growth factor-beta is inhibited in transgenic apolipoprotein(a) mice. Nature 1994; 370:460-2.

Gresser, I. Biological effect of interferons. J. Invest. Dermatol. 1990; 95: 66S-71S.

Hall, F.L., Benya, P.D., Padilla, S.R., Carbonaro-Hall, D., Williams, R., Buckley, S., and Warburton, D. Transforming growth factor-beta type-II receptor signalling: intrinsic/associated casein kinase activity, receptor interactions and functional effects of blocking antibodies. Biochemical Journal 1996; 316(1): 303-10.

Hebda, P.A. Stimulatory effects of transforming growth factor-beta and epidermal factor on epidermal cell outgrowth from porcine skin explant culture. Journal of Investigative Dermatology 1988; 91(5): 440-5.

Ho, Y-S. Transgenic models for the study of lung biology and disease. Am. J. Physiol. 1994; 266: L319-53.

Hoehn, P., Goedert, S., Germann, T., Koelsch, S., Jin, S., Palm, N., Ruede, E., and Schmitt, E. Opposing effects of TGF-β2 on the Th1 cell development of naïve $CD4^+$ T cells isolated from different mouse strains. Journal of Immunology 1995; 155(3): 1349-60.

Hori, M., Kamijo, R., Takeda, K., and Nagumo, M. Down-regulation of c-myc expression by tumor necrosis factor-α in combination with transforming growth factor-β or interferon-γ with concomitant inhibition of proliferation in human cell lines. Journal of Interferon Research 1994; 14(2): 49-55.

Hunter, C.A., Bermudez, L., Beernink, H., Waegell, W., and Remington, J.S. Transforming growth factor-beta inhibits interleukin-12-induced production of interferon-γ by natural killer cells: a role for transforming growth factor-beta in the regulation of T-cell-independent resistance to *Toxoplasma gondii*. European Journal of Immunology 1995; 25(4):994-1000.

Hynes, R.O. Integrins: a family of cell surface receptors. Cell 1987; 48: 549-54.

Ignotz, R. and Massague, J. Transforming growth factor-beta stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matix. J. Biol. Chem. 1986; 261: 4337-45.

Kahari, V.-M., Chen, Y.Q., Bashir, M.M., Rosenbloom, J., and Uitto, J. Tumor necrosis factor-α regulates human elastin gene expression: evidence for the role of AP-1 in the suppression of promoter activity. J. Biol. Chem. 1992; 267: 26134-41.

Karpus, W.J. & Swanborg, R.H. $CD4^+$ suppressor cells inhibit the function of the effector cells of expermental autoimmune encephalomyelitis through a mechanism involving transforming growth factor-beta. J. Immunol. 1991; 146:1163-8.

Katz, A.B. and Taichmann, L.B. Epidermis as a secretory tissue: an in vitro tissue model to study keratinocyte secretion. J. Invest. Dermatol. 1994; 102: 55-60.

Kehrl, J.H., Wakefield, L.M., Roberts, A.B., Jakowlew, S., Alvarez-Mon, M., Derynck, R., Sporn, M.B., and Fauci, AS. Production of transforming growth factor beta by human T lymphocytes and its potential role in the regulation of T cell growth. J. Exp. Med. 1986; 163: 1037-50.

Kim, S.-J., Angel, P., Lafyatis, R., Hattori, K., Kim, K.Y., Sporn, M.B., Karin, M., and Roberts, A.B. Autoinduction of transforming growth factor β1 is mediated by the AP-1 complex. Molecular and Cellular Biology 1990; 10(4): 1492-7.

Kim, S-J., Glick, A., Sporn, M.B., and Roberts, A.B. Characterization of the promoter region of the human transforming growth factor-beta 1 gene. J. Biol. Chem. 1989; 264(1): 402-8.

Kojima, S and Rifkin, D.B. Mechanism of retinoid-induced activation of latent transforming growth factor-β in bovine endothelial cells. J Cell Physiol 1993; 155: 323-332.

Kojima, S. Kiyomitsu, N., Rifkin, D.B. Requirement for transglutaminase in the activation of latent transforming growth factor-β in bovine endothelial cells. J. Cell Biol. 1993; 121: 439-48.

Kondo, S., Kooshesh, F., and Sauder, D.N. Penetration of keratinocyte-derived cytokines into basement membrane. Journal of Cellular Physiology 1997; 171: 190-5.

Kopp, J.B., Factor, V.M., Mozes, M., Nagy, P., Sanderson, N., Bottinger, E.P., Klotman, P.E., and Thorgeirsson, S.S. Transgenic mice with increased plasma levels of TGF-β1 develop progressive renal disease. Laboratory Investigation 1996; 74(6): 991-1003.

Krummel, T.M., Michna, B.A., Thomas, B.L., Sporn, M.B., Nelson, J.M., Salzberg, A.M., Cohen, I.K., and Diegelmann, R.F. Transforming growth factor beta (TGF-β) indices fibrosis is a fetal wound model. J. Pediatr. Surg. 1988; 23(7): 647-52.

Kulkarni, A.B., Huh, C.H., Becker, D., Geiser, A., Lyght, M., Flanders, K.C., Roberts, A.B., Sporn, M.B., Ward, J.M., and Karlsson, S. Transforming growth factor-beta 1 null mutation in mice causes excessive inflammatory response and early death. Proc. Natl. Acad. Sci. USA 1993; 90: 770-4.

Kulozik, M., Hogg, A., Lankat-Buttgereit, B., and Krieg, T. Co-localization of transforming growth factor beta 2 with alpha 1(I) procollagen mRNA in tissue sections of patients with systemic sclerosis. J. Clin. Invest. 1990; 86(3): 917-22

Kuruvilla, A.P., Shah, R., Hochwald, G.M., Liggitt, H.D., Palladino, M.A., and Thorbecke, G.J. Protection effect of transforming growth factor-beta 1 on experimental autoimmune diseases in mice. Proc. Natl. Acad. Sci USA 1991; 88: 2918-21.

Lawn, R.M., Pearle, A.D., Kunz, L.L., Rubin, E.M., Reckless, J., Metcalfe, J.C., and Grainger, D.J. Feedback mechanism of focal vascular lesion formation in transgenic apolipoprotein(a) mice. J. Biol. Chem. 1996; 271(49): 31367-71.

Letterio, J.J. and Roberts, A.B. Transforming growth factor-β1-deficient mice: identification of isoform-specific activities in vivo. Journal of Leukocyte Biology 1996; 59: 769-74.

Letterio, J.J., Geiser, A.G., Kulkarni, A.B., Roche, N.S., Sporn, M.B., and Roberts A.B. Maternal rescue of transforming growth factor-β1 null mice. Science 1994; 264: 1936-8.

Li, X.-F., Takiuchi, H., Zou, J.P., Katagiri, T., Yamamoto, N., Nagata, T., Ono, S., Fujiwara, H., and Hamaoka, T. Transforming growth factor-β1 mediated immunosuppression in the tumor-bearing state: enhanced production of TGF-β and a progressive increase in TGF-β susceptibility of anti-tumor $CD4^+$ T cell function. Jpn. J. Cancer Res. 1993; 84: 315-25.

Liblau, R.S., Singer, S.M., and McDevitt, H.O. Th1 and Th2 $CD4^+$ T cells in the pathogenesis of organ-specific autoimmune diseases. Immunology Today 1995; 16(1): 34-8.

Lin, H.Y., Wang, X-F., Ng-Eaton, E., Weinberg, R.A., and Lodish, H.F. Expression cloning of TGF-β type II receptor, a functional transmembrane serine/threonine kinase. Cell 1992; 68: 775-85.

Lin, J.Y., Sequin, R., Keller, K., and Chadee, K. Transforming growth factor-beta 1 primes macrophages for enhanced expression of the nitric oxide synthetase gene for nitric oxide dependent cytotoxicity against *Entamoeba histolytica*. Immunology 1995; 85(3):400-7.

Lin, R.Y., Sullivan, K.M., Argenta, P.A., Meuli, M., Lorenz, H.P., and Adzick, N.S. Exogenous transforming growth factor-beta amplifies its own expression and induces scar formation in a model of human fetal skin repair. Annals of Surgery 1995; 222(2): 146-54.

Lindsey, J.R., et al. Infectious Diseases of Mice and Rats (National Academy Press, Washington, DC) 1991.

Lopez-Castilla, F., Wrana, J.L., and Massague, J. Betaglycan presents ligand to TGF-β signalling receptor. Cell 1993; 73: 1435-44.

Lorenz, H.P., Lin, R.Y., Longaker, M.T., and Adzick N.S. The fetal fibroblast: the effector cell of scarless fetal skin repair. Plast. Reconstr. Surg. 1995; 96: 1251-61.

Lyons, R.M. and Moses, H.L. Transforming growth factors and the regulation of cell proliferation. Eur. J. Biochem. 1990; 187:467-73.

Lyons, R.M., Gentry, L.E., Purchio, A.F., and Moses, H.L. Mechanism of activation of latent recombinant transforming growth factor-β1 by plasmin. Journal of Cell Biology 1990; 110(4): 1361-7.

Lyons, R.M., Keski-Oja J., and Moses, H.L. Proteolytic activation of latent transforming growth factor-β from fibroblast-conditioned medium. Journal of Cell Biology 1988; 106(5): 1659-65.

Ma, D. & Niederkorn, J.Y. Transforming growth factor-beta down-regulates major histocompatibility complex class I antigen expression and increases the susceptibility of uveal melanoma cells to natural killer cell-mediated cytolysis. Immunology 1995; 86(2): 263-9.

Maeda, H. & Shiraishi, A. TGF-β contributes to the shift toward Th2-type responses through direct and IL-10-mediated pathways in tumor-bearing mice. Journal of Immunology 1996; 156(1): 73-8.

McCartney-Francis, N., Mizel, D., Wong, H., Wahl, L.M., and Wahl, S.M. TGF-β regulates production of growth factors and TGF-β by human peripheral blood monocytes. Growth Factors 1990; 4: 27-35.

Miller, A., Lider, O., Roberta, A., Sporn, M.B., and Weiner, H.L. Suppressor T cells generated by oral tolerization to myelin basic protein suppresses both in vitro and in vivo immune responses by the release of transforming growth factor-beta after antigen-specific triggering. Proc. Natl. Acad. Sci. USA 1992; 89: 421-5.

Mossman, T.R. & Coffman, R.L. Th1 and Th2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu. Rev. Immunol. 1989; 7:145-73.

Mustoe, T.A., Pierce, G.F., Thomason, A., Gramates, P., Sporn, M.B., and Deuel, T.F. Accelerated healing of incisional wounds in rats induced by transforming growth factor-β. Science 1987; 237: 1333-6.

Nunes, I., Gleizes, P.E., Metz, C.N. and Rifkin, D.B. Latent transforming growth factor-beta binding protein domains invovled in activation and transglutaminase-dependent cross-linking of latent transforming growth factor-beta. Journal of Cell Biology 1997; 13(5): 1151-63.

O'Connor-McCourt, M.D., and Wakefield, L.M. Latent transforming growth factor-β in serum: a specific complex with alpha$_2$macroglobulin. J. Biol. Chem. 1987; 262: 14090-14099.

Ohtsuki, M., Tomic-Canic, M., Freedberg, I.M., and Blumenberg, M. Nuclear proteins involved in transcription of the human K5 keratin gene. Journal of Investigative Dermatology 1992; 99(2): 206-15.

Okubo, H., Ishibashi, H., Shibata, J., Tsuda-Kawamura, K., and Yanase, T. Distribution of alpha$_2$macroglobulin in normal, inflammatory, and tumor tissues in rats. Inflammation 1984; 8: 171-9.

Okuda, S., Languino, L.R., Ruoslahti, E., and Border, W.A. Elevated expression of transforming growth factor-beta and proteoglycan production in experimental glomerulonephritis. Possible role in expansion of the mesangial extracellular matrix. J. Clin. Invest. 1990; 86(2): 453-63.

Oursler, M.J., Riggs, B.L., and Spelberg, T.C. Glucocorticoid-induced activation of latent transforming growth factor-beta by normal human osteoblast-like cells. Endocrinology 1993; 133: 2187-96.

Pennica, D., Nedwin, G.E., Hayflick, J.S., Seeburg, P.H., Derynck, R., Palladino, M.A., Kohr, W.J., Aggarwal, B.B., and Goeddel, D.V. Human tumor necrosis factor: precursor structure, expression and homology to lymphotoxin. Nature 1984, 312: 724-9.

Pierce, G.F., Mustoe, T.A., Lingelbach, J., Masakowski, V.R., Griffin, G.L., Senior, R.M., and Deuel, T.F. Platelet-derived growth factor and transforming growth factor-β enhance tissue repair activities by unique mechanisms. The Journal of Cell Biology 1989; 109: 429-40.

Piscatelli, S.J., Michaels, B.M., Gregory, P., Jennings, R.W., Longaker, M.T., Harrison, M.R., and Siebert, J.W. Fetal fibroblast contraction of collagen matrices in vitro: the effects of epidermal growth factor and transforming growth factor-β. Ann. Plast. Surg. 1994; 33: 38-45.

Planchon, S.M., Martins, C.A., Guerrant, R.L., and Roche, J.K. Regulation of intestinal epithelial barrier function by TGF-β1. Evidence for its role in abrogating the effect of a T cell cytokine. Journal of Immunology 1994; 153(12): 5730-9.

Quaglino, D. Jr., Nanney, L.B., Kennedy, R., and Davidson, J.M. Transforming growth factor-beta stimulates wound healing and modulates extracellular matrix gene expresion in pig skin. I. Excisional wound model. Laboratory Investigation 1990; 63(3): 307-17.

Racke, M.K., Canella, B., Albert, P., Sporn, M., Raine, C.S., and McFarlin, D.E. Evidence of endogenous regulatory function of TGF-β 1 in experimental allergic encephalomyelitis. Internatl. Immunol. 1992; 4: 615-20.

Rappaport, H., Khalil, A., Halle-Pannenko, O., Pritchard, L., Dantchev, D., and Mathe, G. Histopathological sequence of events in adult mice undergoing lethal graft-versus-host reaction developed across H-2 and/or non-H-2 histocompatibility barriers. Am. J. Pathol. 1979; 96(1): 121-42.

Roberts, A.B., and Sporn, M.B. Physiological actions and clinical applications of transforming growth factor-beta. Growth Factors 1993; 8: 1-9.

Roberts, A.B., Lamb, L.C., Newton, D.L., Sporn, M.B., DeLarco, J.E., and Todaro, G.J. Transforming growth factors: isolation of polypeptides from virally and chemically transformed cells by acid/ethanol extraction. Proc. Natl. Acad. Sci. USA 1980; 77(6) : 3494-8.

Roberts, A.B., Sporn, M.B., Assoian, R.K., Smith, J.M., Roche, N.S., Wakefield, L.M., Heine, U.I., Liotta, L.A., Falanga, V., Kehrl, J.H., and Fauci, A.S. Transforming growth factor type β: rapid induction of fibrosis and angiogenesis in vivo and stimulation of collagen formation in vitro. Proc. Natl. Acad. Sci. USA 1986; 83: 4167-71.

Robey, P.G., Young, M.F., Flanders, K.C., Roche, N.S., Kondaiah, P., Reddi, A.H., Termine, J.D., Sporn, M.B., and Roberts, A.B. Osteoblasts synthesize and respond to transforming growth factor type beta (TGF-β) in vitro. J. Cell. Biol. 1987; 105:457-63.

Romagnani, S. Biology of human Th1 and Th2 cells. Journal of Clinical Immunology 1995; 15(3): 121-9.

Romagnani, S. Lymphokine production by human T cells in disease states. Annu. Rev. Immunol. 1994; 12: 227-257.

Romagnani, S. Th1 and Th2 in human diseases. Clinical Immunology and Immunopathology 1996; 80(3 part 1): 225-35.

Rossi, P., Karsenty, G., Roberts, A.B., Roche, N.S., and Sporn, M.B. A nuclear factor 1 binding site mediates the transcriptional activation of a type I collagen promoter by transforming growth factor-beta. Cell 1988; 52: 405-15.

Rotello, R.J., Leiberman, R.C., Purchio, A.F., and Gerschenson, L.E. Coordinated regulation of apotosis and cell proliferation by transforming growth factor β1 in cultured uterine epithelial cells. Proc. Natl. Acad. Sci. 1991; 88: 3412-5.

Sachs, L. The control of hematopoiesis and leukemia: from basic biology to the clinic. Proc. Natl. Acad. Sci. USA 1996; 93: 4742-9.

Sad, S. & Mossman, T.R. Single IL-2-secreting precursor CD4 T cell can develop into either Th1 or Th2 cytokine secretion phenotype. Journal of Immunology 1994; 153(8): 3514-22.

Samuel, S.K., Hurta, R.A., Spearman, M.A., Wright, J.A., Turley, E.A., and Greenberg, A.H. Autocrine induction of tumor protease production and invasion by metallothionein-regulated TGF-β1 (Ser 223, 225). EMBO J. 1993; 11(4): 1599-1605.

Sanderson, N., Factor, V., Nagy, P., Kopp, J., Kondaiah, P., Wakefield, L., Roberts, A.B., Sporn, M.B., amd Thorgeirsson, S.S. Hepatic expression of mature transforming growth factor-β1 in transgenic mice results in multiple tissue lesions. Proc. Natl. Acad. Sci. USA 1995; 92: 2272-76.

Sato, Y. and Rifkin, D.B. Inhibition of endothelial cell movement by pericytes and smooth muscle cells: activation of a latent transforming growth factor-β1-like molecule by plasmin during co-culture. J. Cell Biol. 1989; 109:309-15.

Sawamura, D., Meng, X., Ina, S., Sato, M., Tamai, K., Hanada, K., and Hashimoto, I. J. Immunol. 1998; 161(10): 5633-9.

Saunders, N.A. & Jetten, A.M. Control of growth regulatory and differentiation-specific genes in human epidermal keratinocytes by interferon gamma. Antagonism by retinoic acid and transforming growth factor-beta 1. J. Biol. Chem. 1994; 269(3): 2016-22.

Schultz-Cherry, S., and Murphy-Ullrich, J.E. The type I repeats of thrombospondin I activate latent transforming growth factor-β. J. Biol. Chem. 1994; 264: 26783-88.

Schultz-Cherry, S., and Murphy-Ullrich, J.E. Thrombospondin causes activation of latent transforming growth factor-beta secreted by endothelial cells by a novel mechanism. J. Cell Biol. 1993; 122: 923-32.

Sellheyer, K., Bickenbach, J.R., Rothnagel, J.A., Bundman, D., Longley, M.A., Krieg, T., Roche, N.S., Roberts, A.B., and Roop, D.R. Inhibition of skin development by overexpression of transforming growth factor β1 in the epidermis of transgenic mice. Proc. Natl. Acad. Sci. USA 1993; 90: 5237-41.

Shah, M., Foreman, D.M., and Ferguson, M.W. Control of scarring in the adult wounds by neutralising antibody to transforming growth factor β. Lancet 1992; 339: 213-4.

Shah, M., Foreman, D.M., and Ferguson, W.J. Neutralization of TGF-β1 and TGF-β2 on exogenous addition of TGF-β3 to cutaneous rat wounds reduces scarring. J. Cell. Sci. 1995; 108: 985-1002.

Shain, S.A., Lin, A.L., Koger, J.D., and Karanganis, A.G. Rat prostrate cancer cells contain functional receptors for the transforming growth factor-beta. Endocrinology 1990; 126:818-25.

Spaccapelo, R., Romani, L., Tonnetti, L., Cenci, E., Mencacci, A., Del Sero, G., Tognellini, R., Reed, S.G., Puccetti, P., and Bistoni, F. TGF-β is important in determining the in vivo patterns of susceptibility or resistance in mice infected with *Candida albicans*. Journal of Immunology 1995; 155(3): 1349-60.

Sporn, M.B., Roberts, A.B., Shull, J.H., Smith, J.M., and Ward, J.M. Polypeptide transforming growth factors isolated from bovine sources and used for wound healing in vivo. Science 1983; 219: 1329-31.

Sporn, M.B., Roberts, A.B., Wakefield, L.M., and Assoian, R.K. Tranforming growth factor-β: biological function and chemical structure. Science 1986, 233(4763):532-5.

Staggers, W.R., Paterson, A.J., and Kudlow, J.E. Sequence of the functional human keratin K14 promoter. Gene 1995; 153: 297-8.

Stegemann, H. and Stalder, K. Determination of hydroxyproline. Clinica. Chim. Acta. 1967; 18: 267-73

Steinert, P.M. & Roop, D.R. Molecular and cellular biology of intermediate filaments. Ann. Rev. Biochem. 1988;57:593-625.

Sullivan K.M., Lorenz, H.P. and Adzick, N.S. The role of transforming growth factor beta in human fetal wound healing. Surg. Forum 1993; 79: 625-6.

Sullivan, K.M., Lorenz, H.P., Meuli, M., Lin, R.Y., and Adzick N.S. A model of scarless human feal wound healing repair is deficient in transforming growth factor beta. J. Pediatr. Surg. 1995; 30: 198-203.

Taipale, J., Lohi, J., Saarinen, J., Kovanen, P.T., and Keski-Oja, J. Human mast cell chymase and leukocyte elastase release latent transforming growth factor-β1 from the extracellular matrix of cultured human epithelial and endothelial cells. Journal of Biological Chemistry 1995; 270 (9): 4689-96.

Taipale, J., Miyazono, K., Heldin, C.H., and Keski-Oja, J. Latent transforming growth factor-β1 associates to fibroblast extracellular matrix via latent TGF-β binding protein. Journal of Cell Biology 1994; 124(1-2): 171-81.

Taipale, J., Saharinen, J., Hedman, K., and Keski-Oja, J. Latent transforming growth factor-β1 and its binding protein are components of extracellular matrix microfibrils. Journal of Histochemistry & Cytochemistry 1996; 44(8): 875-89.

Theodoresu, D., Bergsma, D., MS, Elshourbagy, N., Sheehan, C., Rieman, D., and Kerbel, R.S. Cloning and overexpression of TGF-β1 cDNA in a mammary adenocarcinoma: in vitro and in vivo effects. Growth Factors 1991; 5(4): 305-16.

Thompson, T.C., Truong, L.D., Timme, T.L., Kadmon, D., McCune, B.K., Flanders, K.C., Scardino, P.T., and Park, S.H. Transgenic models for the study of prostrate cancer. Cancer 1993; 71(3 suppl): 1165-71.

Tomic-Canic, M., Sunjevaric, I., Freedberg, I.M., and Blumberg, M. Identification of the retinoic acid and thyroid hormone receptor-responsive element in the human K14 keratin gene. Journal of Investigative Dermatology 1992; 99(2): 206-15.

Tredget, E.E., Nedelec, B., Scott, P.G., and Ghahary, A. Hypertrophic scars, keloids and contractures. SCNA 1997; 77(3): 701-730.

Tuan, T.-L. and Nichter, L.S. The molecular basis of keloid and hypertrophic scar formation. Molecular Medicine Today 1998; Jan: 19-24.

Turksen, K., Kupper, T., Degenstain, L., Williams, I., and Fuchs, E. Interleukin 6: insights to its function in skin by overexpression in transgenic mice. Proc. Natl. Acad. Sci. 1992; 89: 5068-72.

Van Obberghen-Schilling, E., Roche, N.S., Flanders, K.C., Sporn, M.B., and Roberts, A.B. Transforming growth factor-beta 1 positively regulates its own expression in normal and transformed cells. The Journal of Biological Chemistry 1988; 263 (16): 7741-6.

Varedi, M., Tredget, E.E., Scott, P.G., Shen, Y.J., and Ghahary, A. Alteration in cell morphology triggers transforming growth factor-β1, collagenase and tissue inhibitor of metalloproteinases-I expression in normal and hypertrophic scar fibroblasts. Journal of Investigative Dermatology 1995; 104: 118-23.

Varga, J. and Jimenez, S.A. Stimulation of normal human fibroblast collagen production and processing by transforming growth factor-beta. Biochem. Biophys. Res. Commun. 1986; 138: 974-80.

Vassar, R., Rosenberg, M., Ross, S., Tyner, A., and Fuchs, E. Tissue-specific and differentiation-specific expression of a human K14 keratin gene in transgenic mice. Cell Biology 1989; 86: 1563-7.

Vodovotz, Y., Bogdan, C., Paik, J., Xie, Q.-W., and Nathan, C. Mechanisms of suppression of macrophage nitric oxide release by transforming growth factor β. J. Exp. Med. 1993; 178: 605-13.

Vrabec, J.T., Schwaber, M.K., Davidson, J.M., et al. Evaluation of basic fibroblast growth factor in tympanic membrane repair. Laryngoscope 1994; 104: 1059-64.

Wahl, S.M. Transforming growth factor β: the good, the bad, and the ugly. The Journal of Experimental Medicine 1994; 180: 1587-90.

Wahl, S.M., Hines, K.L., Christ, M., McCartney-Francis, N., Kulkarni, A., Karlsson, S., Furcht, L, and McCartney, J.B. Adhesion, recruitment, and activation of mononuclear phagocytes in inflammation. J. Leuko. Biol. 1993; 54(Suppl): 74-81.

Wahl, S.M., Hunt, D.A., Wakefield, N.M., McCartney-Francis, N., Wahl, L.M., Roberts, A.B., and Sporn, M.B. Transforming growth factor type β induces monocyte chemotaxis and growth factor production. Proc. Natl. Acad. Sci. USA 1987; 83: 4167-71.

Wakefield, L.M., Smith, D.M., Flanders, K.C., and Sporn, M.B. Latent transforming growth factor from human platelets. J. Biol. Chem. 1988; 263: 2646-54.

Wakefield, L.M., Winokur, T.S., Hollands, R.S., Christopherson, K., Levinson, A.D., and Sporn, M.B. Recombinant latent transforming growth factor β1 has a longer plasma half-life in rats than active transforming growth factor β1, and a different tissue distribution. J. Clin. Invest. 1990; 86: 1976-84.

Wang, X., Zinkel, S., Polonsky, K., and Fuchs, E. Transgenic studies with a keratin promoter-driven growth hormone transgene: prospects for gene therapy. Proc. Natl. Acad. Sci. USA 1997; 94: 219-26.

Whitby, D.J. and Ferguson, M.W. Immunohistochemical localization of growth factors in fetal wound healing. Dev. Biol. 1991; 147: 207-15.

Wrana, J.L., Attisano, L., Wieser, R., Ventura, F., and Massague, J. Mechanism of activation of TGF-β receptor (receptor constituitively active). Nature 1994; 370:341-7.

Wrana, J.L., Wang, X-F., and Massague, J. TGF-β signals through a heteromeric protein kinase receptor complex. Cell 1992; 71: 1003-14.

Wu, L., Siddiqui, A., Morris, D.E., Cox, D.A., Roth, S.I., and Mustoe, T.A. Transforming growth factor β3 (TGF-β3) accelerates wound healing without alteration of scar prominence. Arch. Surg. 1997; 132: 753-60.

Yamaguchi, Y., Mann, D.M., and Ruoslahti, E. Negative regulation of transforming growth factor-beta by the proteoglycan decorin. Nature 1990; 346(6281): 218-4.

Yang, C-C., Lin, S-D., and Yu, H-S. Effect of growth factors on dermal fibroblast contraction in normal and hypertrophic scar. Journal of Dermatological Science 1997, 14: 162-9.

Yang, L., Qiu, C.X., Ludlow, A., Ferguson, M.W., amd Brunner, G. Active transforming growth factor-β in wound repair. American Journal of Pathology, 1999; 154(1): 105-11.

Yorchenco, P.D. and Schittny, J.C. Molecular architecture of basement membranes. FASEB J. 1990; 4: 1577-90.

Younai, S., Venters, G., Vu, S., Nichter, L., Nimni, M.E., and Tuan, T.L. Role of growth factors in scar contraction: an in vitro analysis. Ann. Plast. Surg. 1996; 36(5): 495-501.

Younai, Y.Y., Nichter, L.S., Wellisz, T., Reinisch, J., Nimni, M.E., and Tuan, T.L. Modulation of collagen synthesis by transforming growth factor-β in keloid and hypertrophic scar fibroblasts. Ann. Plast. Surg. 1994; 33: 148-51.

Zhang, M-C., Giro, M., Quaglino, D., and Davidson, J.M. Transforming growth factor-β reverses a posttranscriptional defect in elastin synthesis in a cutis laxa skin fibroblast strain. J. Clin. Invest. 1995; 95; 986-94.

Zhang, X., Giangreco, L., Broome, H.E., Dargan, C.M., and Swain, S.L. Control of CD4 effector fate: transforming growth factor-beta 1 and interleukin 2 synergize to prevent apoptosis and promote effector expansion. Journal of Experimental Medicine 1995; 182(3): 699-709.

Zhou, S., Kinzler, K.W., and Vogelstein, B. Going mad with Smads. New Engl. J. Med. 1999; 341(15): 1144-6.

pG3Z-K14:TGF-β Construct

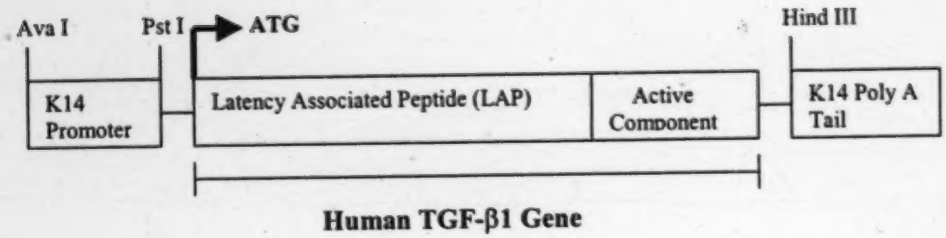


Figure 1.

Plasmid construct used to make the transgene. The human TGF-β1 gene was ligated to the keratin 14 promoter and the keratin 14 poly A tail to direct transgene expression to the keratinocytes. The latency associated peptide was included to produce a latent TGF-β1 transgene product as opposed to a constitutively active TGF-β1. Restriction sites are shown and the arrow denotes the start site of transcription (ATG).

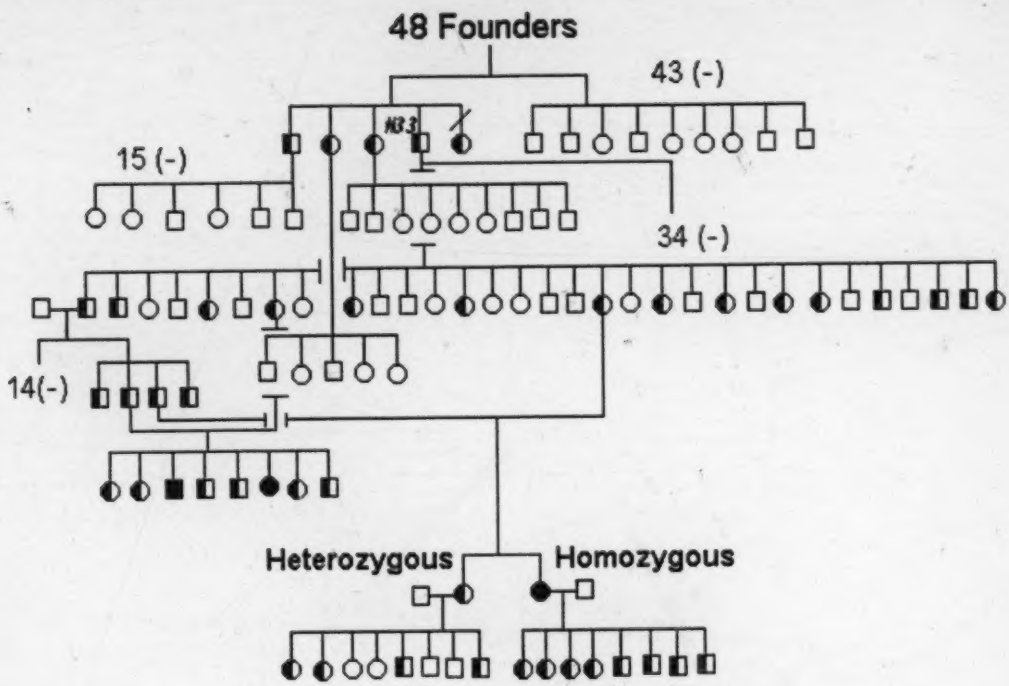
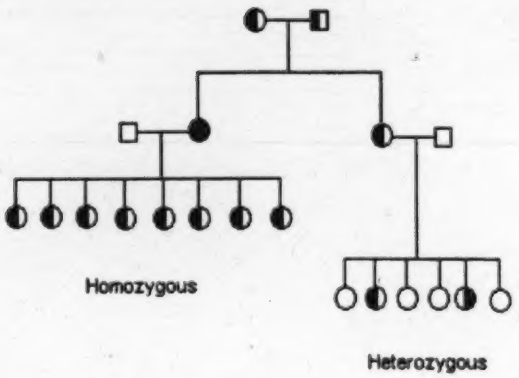


Figure 2.

Transgenic pedigree. From 48 founders, 5 mice tested positive for the human TGF-β1 transgene by Southern blotting. These mice were bred to normal animals to test the stability of the transgene and the ability of the founder mice to pass it along to its progeny. Only one founder mouse (Fo#33) produced viable progeny that possessed the transgene. From this founder, homozygous and heterozygous mice were produced and subsequently used in our experiments.

Panel A



Panel B

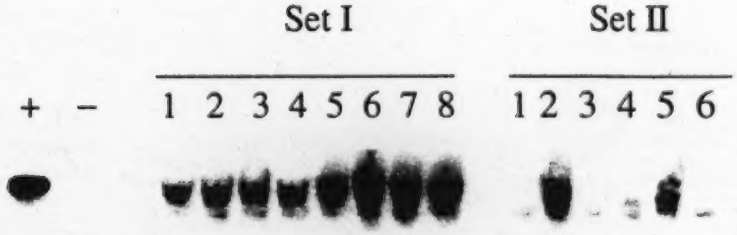


Figure 3.

Panel A. Generation of homozygous animals. The Southern blotting of the F1 progeny from the founder mouse revealed that the integration of the transgene was autosomal and progeny inherited the transgene in a heterozygous fashion. The production of homozygous animals must be generated from the mating of two heterozygous animals where 25% of the progeny from this mating will be homozygous. Testing for homozygous animals was based on the fact that 100% of the offspring from a mating between a homozygous and a normal mouse will genotypically be heterozygous for the transgene which can be detected by Southern blotting. **Panel B. Southern blotting results of progeny from testing of potential homozygous animals.** Set I and set II are potential homozygous animals (from two heterozygous parents) mated with normal mice. Set I shows that all progeny possess the transgene and therefore the tested parent is a homozygous animal for the K14-hTGF-β1 transgene. Any progeny from a homozygous testing pair that gives a negative Southern blotting result quickly rules out the chance that the parent in question is a homozygous animal (as in set II). +, positive control with TGF-β DNA; -, negative control with collagen type I DNA.

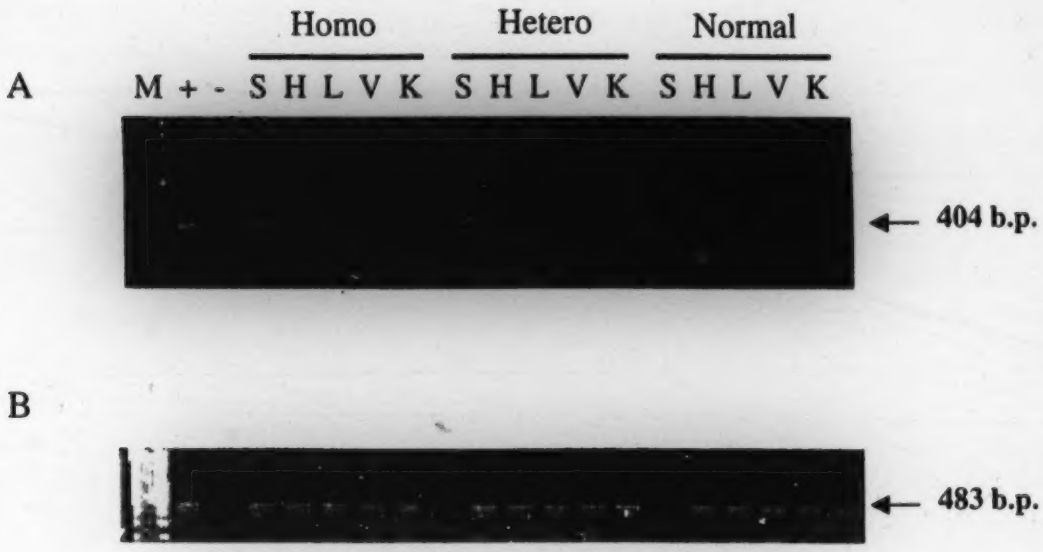


Figure 4.

Reverse transcription polymerase chain reaction (RT-PCR). RT-PCR of mRNA from different organs using primers developed for the detection of human TGF-β1 product of 404 base pairs in length (panel A) or mouse TGF-β1 of 483 b.p. (panel B). Panel A shows the specificity of transgene expression as human TGF-β1 mRNA is only detected in the skin of homozygous and heterozygous animals and not in any other organs tested. Panel B shows endogenous TGF-β1 is expressed in all of the organs tested. For complete account of primer sequences and sample handling, see Materials and Methods. M, 100 base pair DNA ladder; +, positive control (hTGF-β1 transgene plasmid DNA); -, negative control (RNA extracted from the skin of homozygous animals not reverse transcribed); S, skin; H, heart; L, lungs; V, liver; K, kidney.

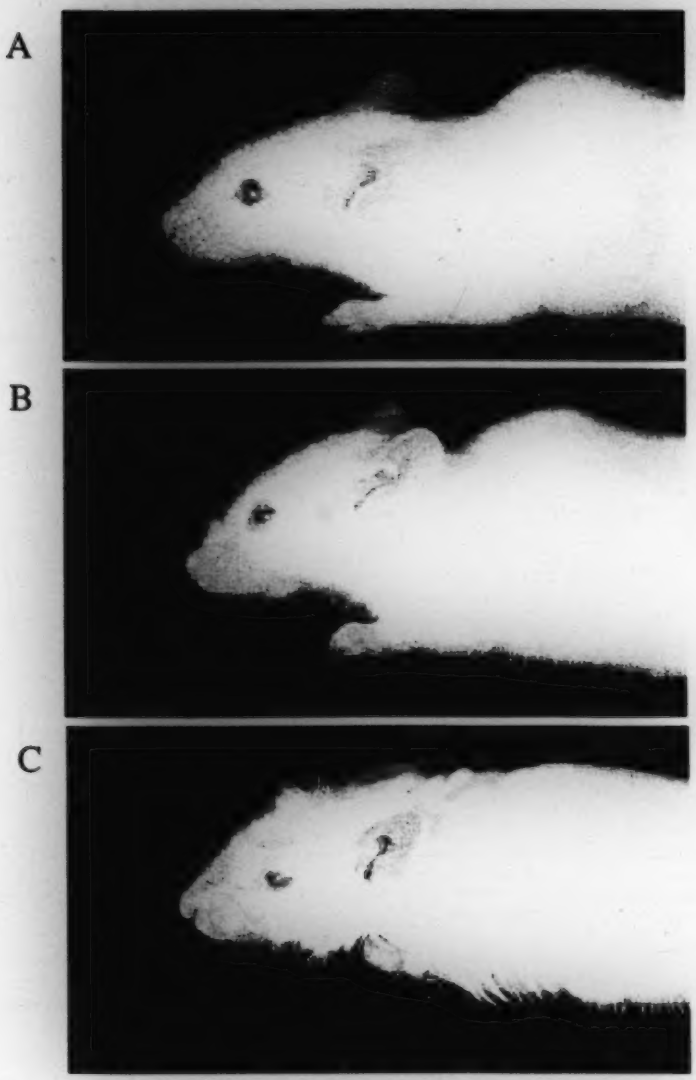


Figure 5.

Phenotypic changes. A) normal mouse, B) heterozygous mouse and C) homozygous mouse. Signs of scruffiness was only observed in the homozygous lines at varying ages of mice (from two months to over a year). Mice shown are age matched.

Panel A

Panel B

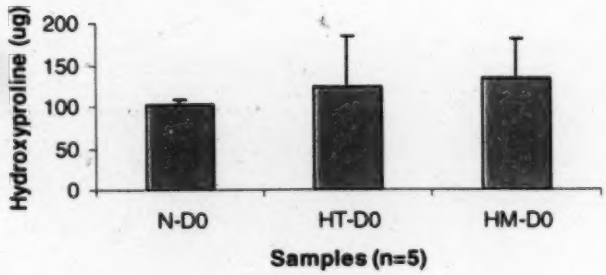


Figure 6.

Panel A. Masson's trichrome staining of skin cross-sections from normal, heterozygous and homozygous animals. Although the homozygous animals appear to possess a thinner dermis, this observation can not be accounted for by the hydroxyproline analysis in the sections (Panel B). **Panel B Hydroxyproline levels in the skin.** Observed is a slight increase in hydroxyproline content on average in the homozygous and heterozygous animals that is not statistically significant (P=0.5717, n=5) compared to normal controls. Error bars denote standard deviation.

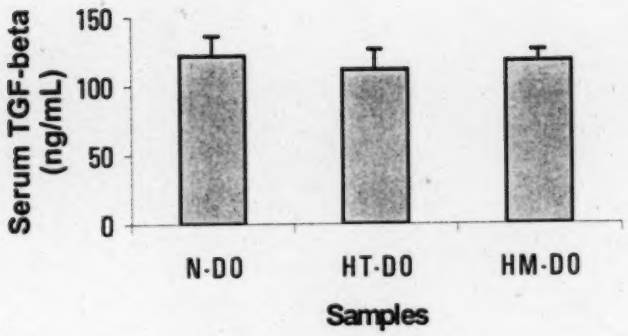


Figure 7.

Serum TGF-β1 protein levels. TGF-β1 serum levels were measured in the different groups of animals. There was no statistical difference in the amount of circulating TGF-β1 between the homozygous (HM-D0), heterozygous (HT-D0) and normal (N-D0) animals. (ANOVA, p=0.2497, n=9). Day 0 refers to a time prior to wounding. Error bars denote standard deviation. Error bars denote standard deviation.

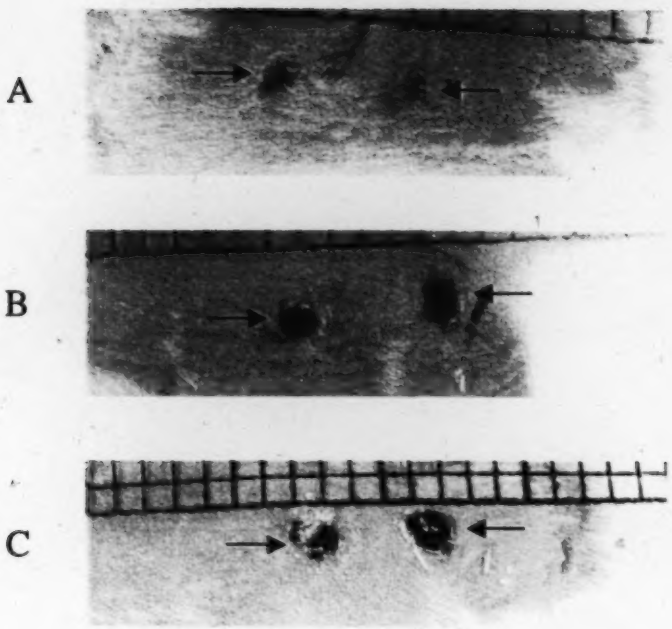


Figure 8.

Wound healing. Biopsy wounds at day 9, where the greatest contrast in wound healing occurred. Wounds from a normal animal (A), heterozygous animal (B) and homozygous animal (C) are compared. The arrows indicate where the wounds are after the removal the scab (if any). Significant differences in re-epithelialization can be observed macroscopically without histological sections.

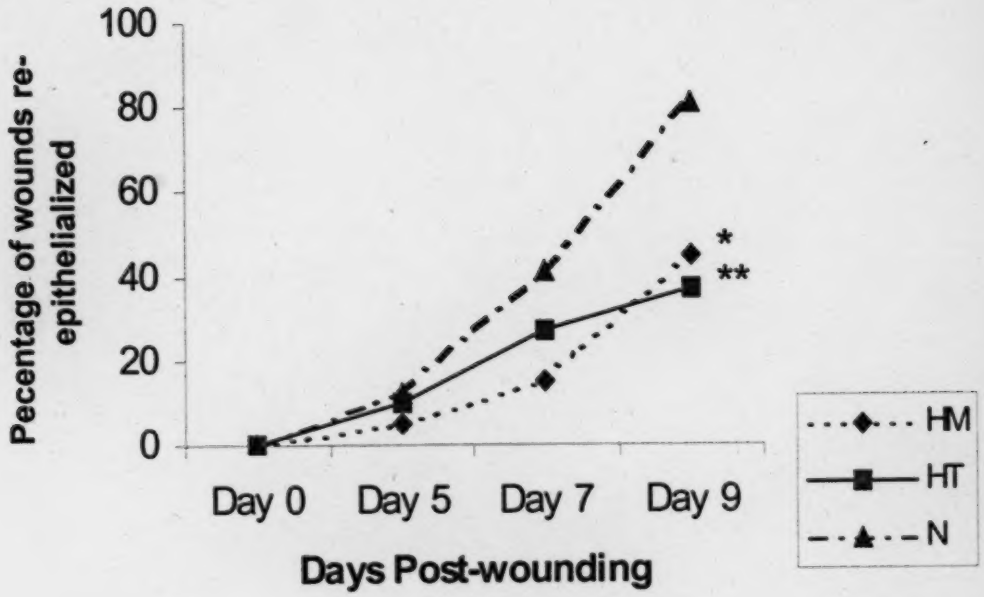
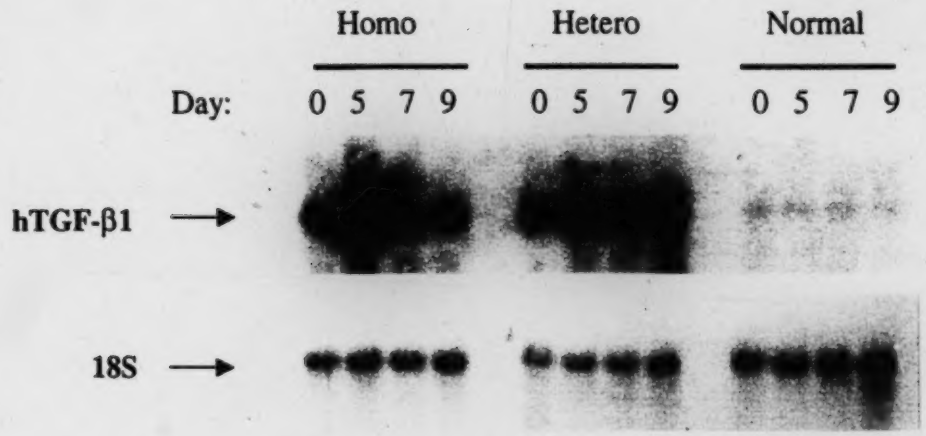


Figure 9.

Wound closure. The percentage of wounds closed at different time points post-wounding is depicted in this graph. At day 5 post-wounding, there is not a statistically significant difference in healing between the three groups of animals. On day 7 post-wounding, there was a significant difference in wound healing between the normal animals (N) and homozygous animals (HM) (Fisher's Exact Test, p=0.015). By day 9 post-wounding, differences were seen between homozygous and normal mice (single asterix (*) indicates p=0.0014, Fisher's Exact Test) and between the heterozygous animals (HT) and normal animals (double asterix (**) indicates p=0.00014, Fisher's Exact Test). There was never any statistical difference in wound re-epithelization between the homozygous and heterozygous animals.

A



B

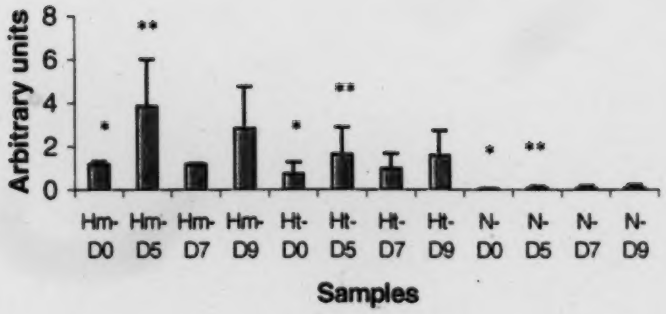
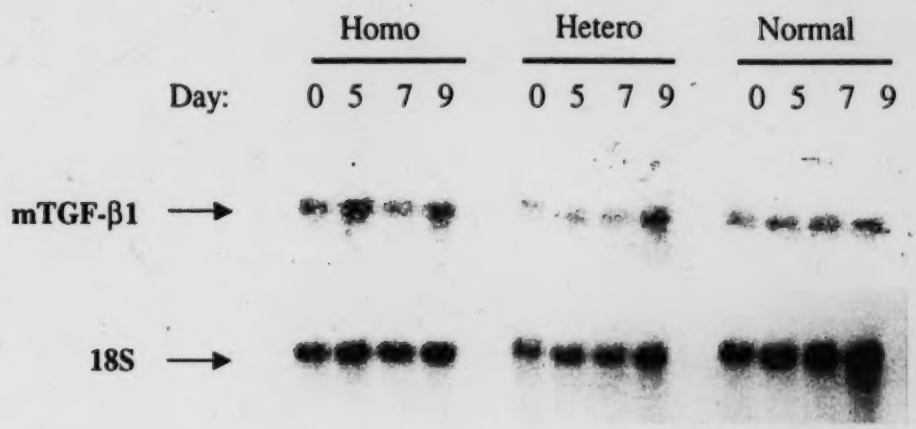


Figure 10.

Northern analysis for human TGF-β1 mRNA expression. Panel A is the northern blot autoradiogram; **panel B** is an attempt at quantitating the results from the northern analysis by assigning arbitrary densitometer units. The maximum level of hTGF-β expression was observed at day 5 post-wounding for both the heterozygous (Hetero) and especially homozygous (Homo) animals. This data represents one set of animals with a different animal at each time point. The arrow denotes TGF-β1 mRNA signal. Error bars represents standard deviation. Single asterix (*) denotes significance between the groups of animals at day 0 (p=0.0241, ANOVA). Double asterix (**) denotes significance at day 5 (p=0.0089, ANOVA).

A



B

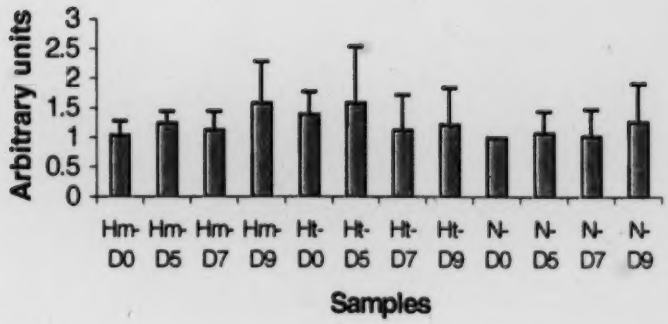
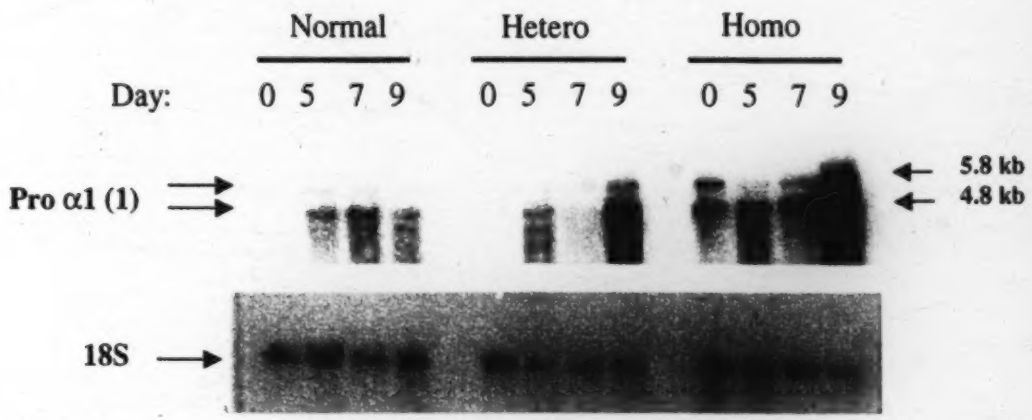


Figure 11.

Northern analysis for mouse TGF-β1 mRNA expression. There does not appear to be any significant difference in endogenous mRNA expression of TGF-β1. **Panel A** is the northern analysis autoradiogram; **panel B** show the results of the northern analysis expressed as arbitrary densitometer units. Error bars indicate the standard deviation.

A



B

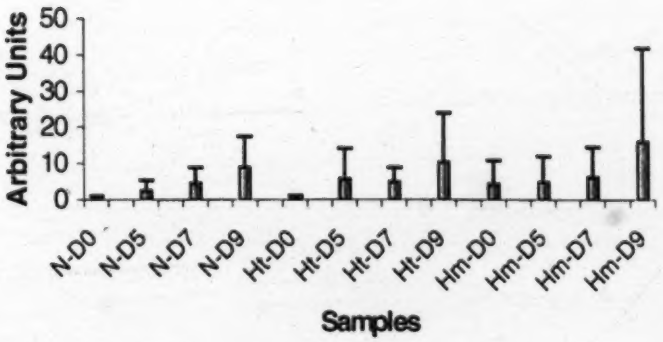
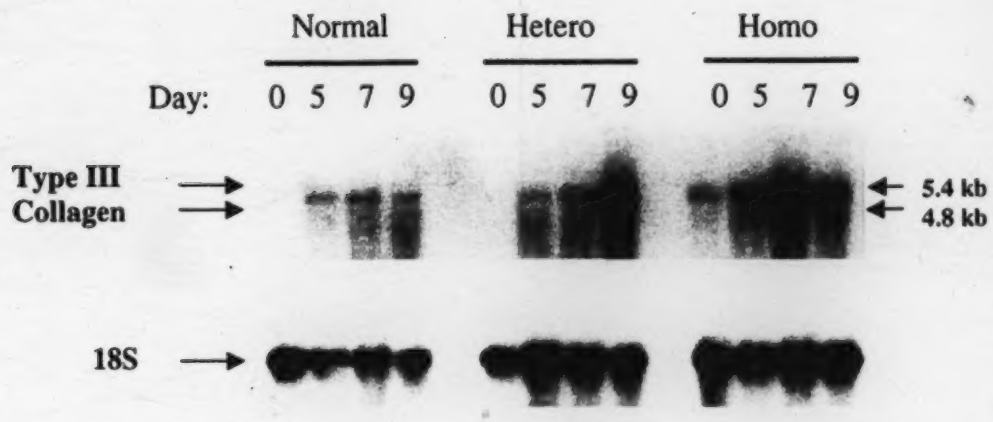


Figure 12.

Northern analysis for pro α1 (typeI) collagen mRNA expression. Increased expression of mRNA was observed in the homozygous (Homo) animals over the heterozygous (Hetero) and normal animals in certain sets of blots, but ultimately was not reflected in the hydroxyproline analysis. Both transcripts of mRNA are seen as indicated by 5.8 kb and 4.8 kb arrows. **Panel A** is the northern analysis autoradiogram; **panel B** show the results of the northern analysis expressed as arbitrary densitometer units.

A



B

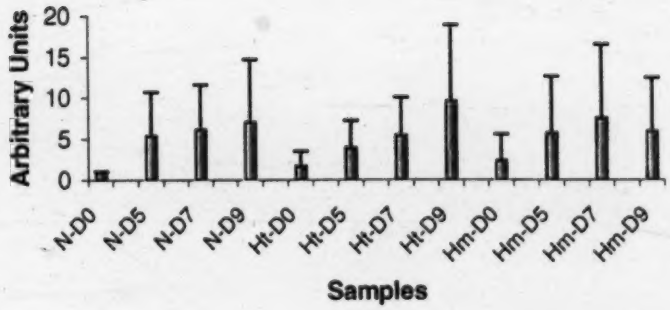


Figure 13.

Northern Analysis for Type III collagen mRNA expression. Only in this set did transgenic animals show increased Type III collagen mRNA production at the later days post-wounding. Peak expression occurred at day 7 for the homozygous and day 9 for the heterozygous animal. **Panel A** is the northern analysis autoradiogram; **panel B** show the results of the northern analysis expressed as arbitrary densitometer units.

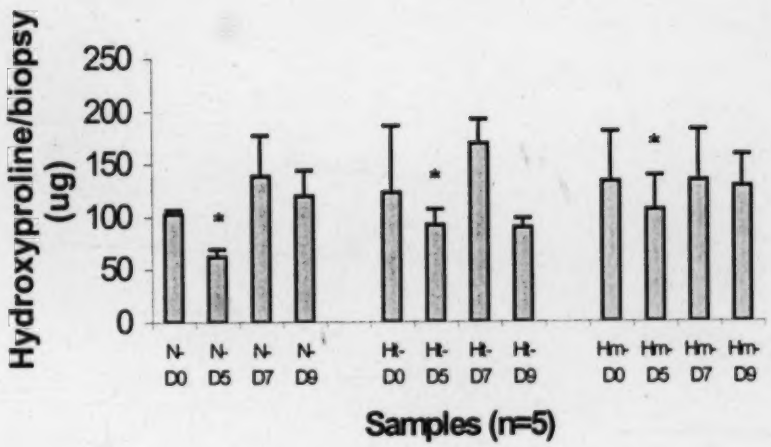


Figure 14.

Hydroxyproline content post-wounding. Depicted here is the average readings of five biopsies from homozygous (Hm), heterozygous (Ht), and normal animals for day 0 (D0), 5 (D5), 7 (D7) and 9 (D9) post-wounding. Single asterix (*) denotes significance between the groups of animals at day 5 (p=0.0170, ANOVA). Error bars indicate standard deviations.

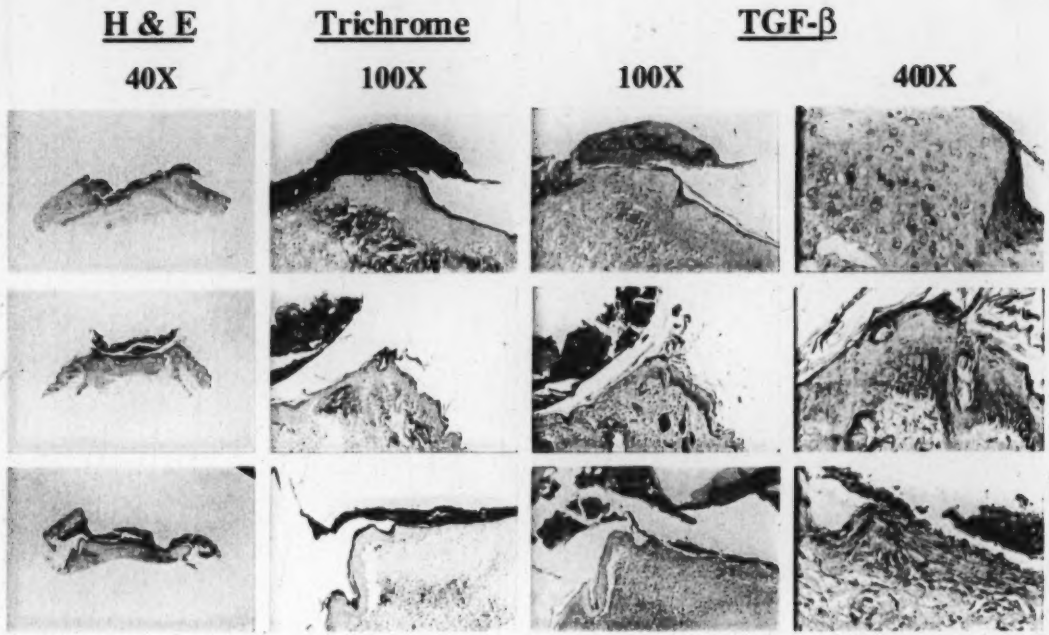


Figure 15.

TGF-β immunohistochemistry at day 5 post-wounding. In the normal H&E staining at 40X magnification, all the wounds are still open. Sections from the normal animals show weak staining for TGF-β only in the advancing portion of the epithelium. The homozygous and heterozygous animals both showed more intense staining in the wound sections here at day 5 post-wounding. Interestingly, TGF-β staining is not limited to the vicinity of the wound, but staining in the basal layer of the epidermis is also observed in the distal portions of the epithelium.

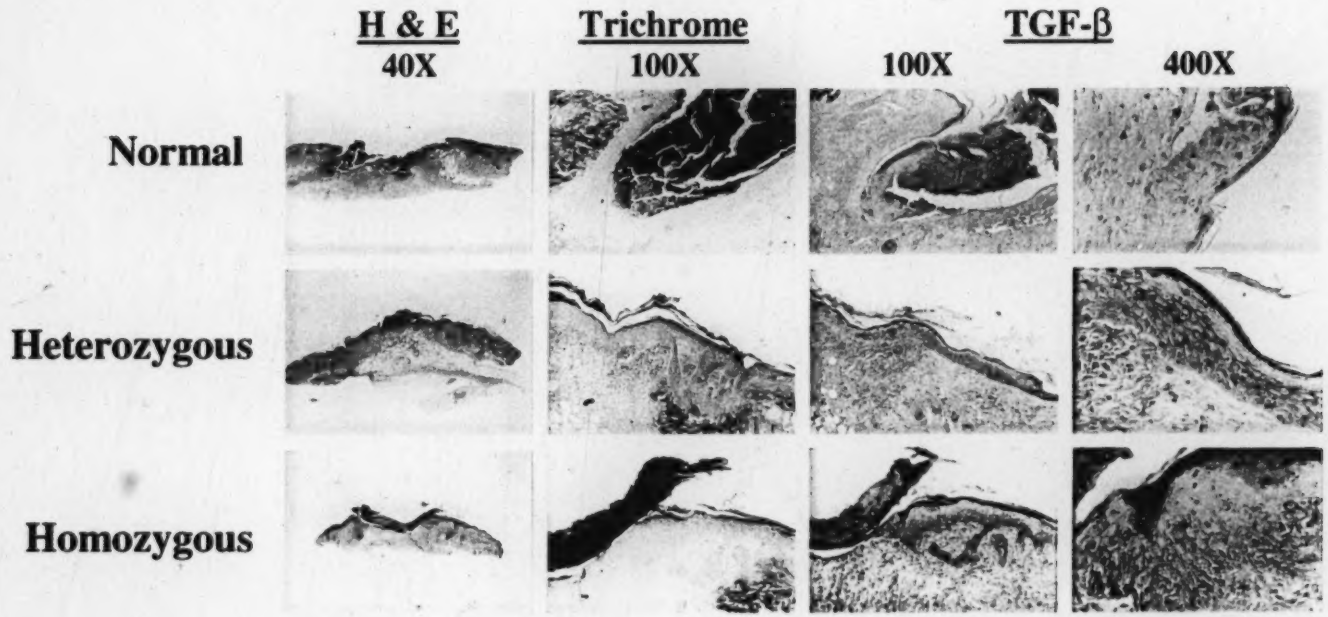


Figure 16.

TGF-β immunohistochemistry at day 7 post-wounding. Most wounds at this time point are still open. TGF-β staining in the normal animals is still restricted to the advancing epithelium, shown here migrating beneath the scab. Compared to the homozygous and heterozygous sections, the normal animals appear to possess the most diffuse staining with the TGF-β antibody. In the homozygous and heterozygous samples, the basal layer is still staining intensely and the distal portions of the epidermis from the wound site are also stained.

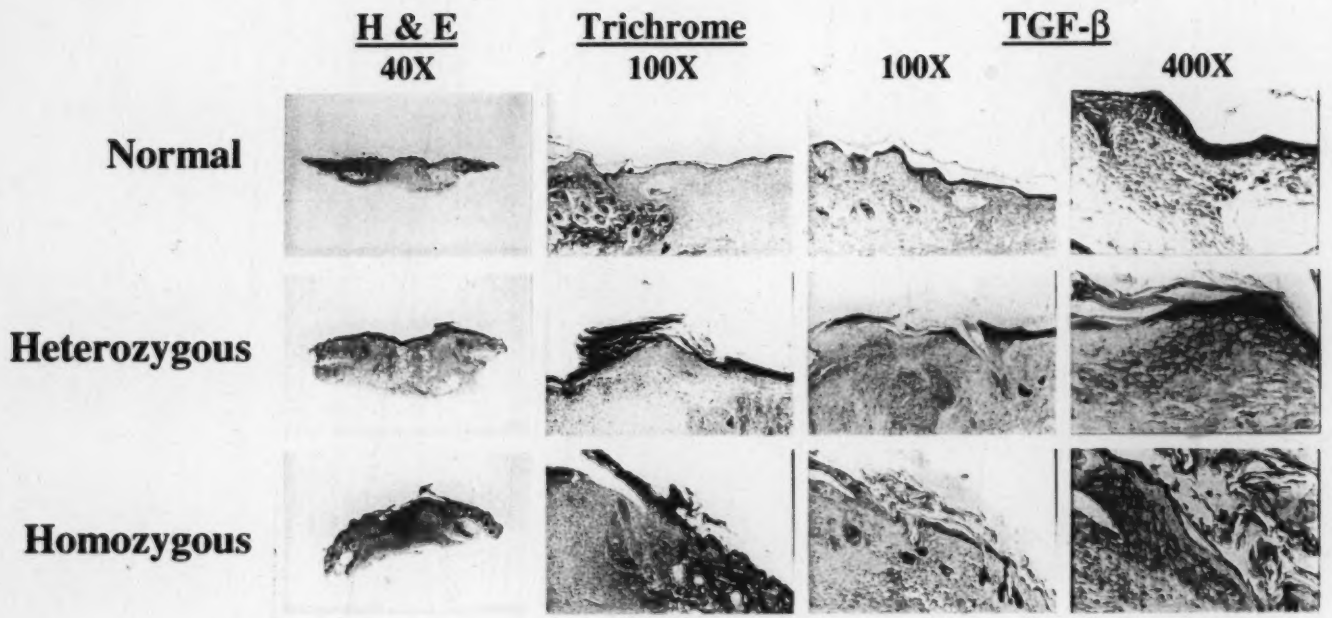


Figure 17.

TGF-β immunohistochemistry at day 9 post-wounding. In the normal H & E staining at 40X magnification, the wound is now closed and keratinization is observed. The heterozygous and homozygous samples have not yet completely re-epithelialized at this time point. TGF-β staining shows intense staining in all three samples, but only in the homozygous and heterozygous samples can staining in the basal keratinocyte layers be observed.

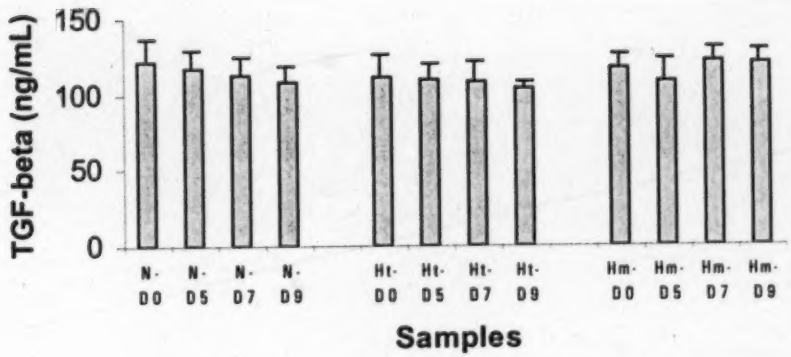
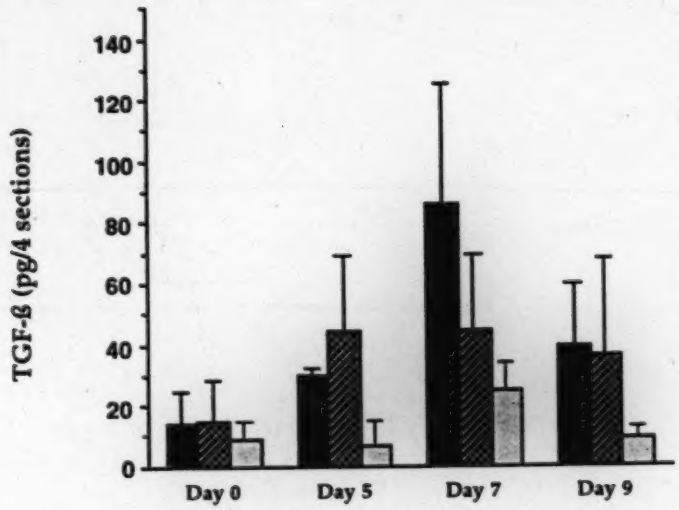


Figure 18.

Serum TGF-β1 levels post-wounding. Serum TGF-β1 levels did not become increased over the wound healing course, indicating a localized effect of the transgene.
Hm, homozygous animal biopsies; Ht, heterozygous animal biopsies; N, normal animal biopsies. Days 0, 5, 7, and 9 post-wounding are indicated as D0, D5, D7 and D9 respectively.

A


TGF-ß (pg/4 sections)
140
120
100
80
60
40
20
0
Day 0
Day 5
Day 7
Day 9

B

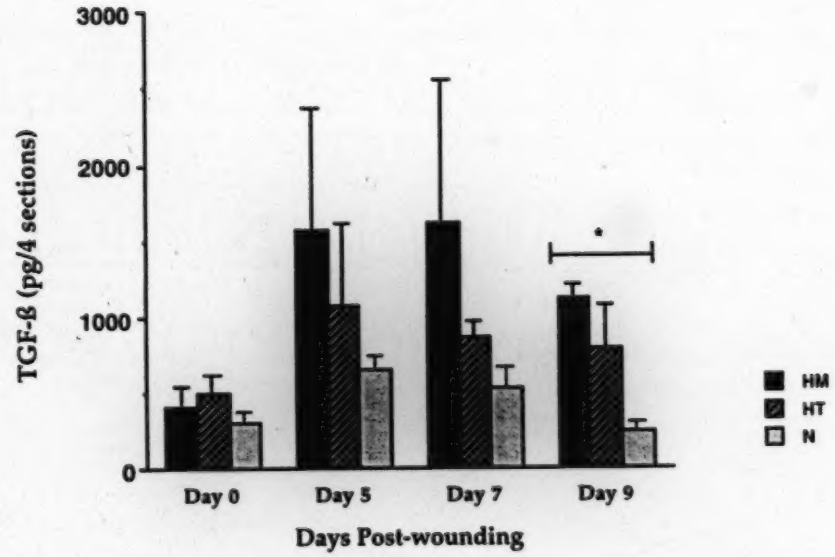

TGF-ß (pg/4 sections)
3000
2000
1000
0
*
Day 0
Day 5
Day 7
Day 9
Days Post-wounding
HM
HT
N

Figure 19.

PAI/L assay for *in vivo* TGF-ß1 levels. Panel A represents the amount of active TGF-ß1 found in the biopsies of the wounds at different days post-wounding. This data suggests that both homozygous and heterozygous transgenic mice produce higher levels of active TGF-ß1 in the wounds during healing. **Panel B** represents the amount of total TGF-ß1 detected in the wounds during healing. Statistical significance was found only at day 9 post-wounding but this data suggests that levels are consistently higher in the transgenic animals with the highest levels found in the homozygous animals after injury. Single asterix (*) denotes significance between animals ($p<0.001$, ANOVA)

Appendix I—Homozygous Breeding

mouse I.D.	total	(+)	(-)
Founders	48	5	43
F1 from Founders	80	15	65
F2 (33-7, 33-8)	31	9	22
Homozygous Breeding (four lines)	85	30	55
Homozygous Testing (30 mice)	264	6 of 30	24 of 30
Total Mice Tested: 508			

Appendix I.

Total mice tested to produce 4 stable homozygous mating pairs from one founder. From 48 founders, only 5 carried the transgene. 80 mice were generated from these 5 founders to establish that only Fo#33 could generate progeny that possessed that transgene. An F2 generation was tested to show the transgene would be stable over several generations. Subsequent mating and testing of homozygous animals involved another 264 animals for a total of 508 mice that were tested by Southern blotting to finally generate a continous supply of homozygous and heterozygous mating pairs from Fo#33.

Appendix II

Human TGF-β1 mRNA Sequence:

hTGF-β1 PCR Product Sequence:

```
   1                                    AGCATAT ATATGTTCTT CAACACATCA
  51  GAGCTCCGAG AAGCGGTACC TGAACCGTG TTGCTCTCCC GGGCAGAGCT
 101  GCGTCTGCTG    AGGCTCA AGTTAAAAGT GGAGCAGCAC GTGGAGCTGT
 151  ACCAGAAATA CAGCAACAAT TCCTGGCGAT ACCTCAGCAA CCGGCTGCTG
 201  GCGGCAGTGG TTGAGCCGTG GAGGGGAAAT TGAGGGCTTT CGCCTTAGCG
 251  CCCACTGCTC C
```

Appendix II.

The PCR product for the human TGF-β1 RT-PCR was electroeluted, precipitated and sent for DNA sequencing to ensure that the bands seen are from transgene expression. Sequencing results show that the homozygous and heterozygous mice are expressing human TGF-β1 and is seen only in the skin of these animals. From this result, we are confident that the transgene is functional and properly targeted to the skin by the K14 promoter. This product was later used to produce probes for northern analysis to observe the mRNA production of hTGF-β1 after wounding. Single lines indicate the primer annealing site; double lines denote PCR product sequence in relation to TGF-β gene sequence. Primer sequences can be found in Materials and Methods.

Appendix III

Mouse TGF-β1 mRNA Sequence

```
   1  GGTGCGCCCC AGCCTGCCTC TTGAGTCCCT CGCATCCCAG GACCCTCTCT
  51  CCCCCGAGAG GCAGATCTCC CTCGGACCTG CTGGCAGTAG CTCCCCTATT
 101  TAAGAACACC CACTTTTGGA TCTCAGAGAG CGCTCATCTC GATTTTTACC
 151  CTGGTGGTAT ACTGAGACAC CTTGGTGTCA GAGCCTCACC GCGACTCCTG
 201  CTGCTTTCTC CCTCAACCTC AAATTATTCA GGACTATCAC CTACCTTTCC
 251  TTGGGAGACC CCACCCCACA AGCCTGCAGG GGCGGGGCCT CCGCATCCCA
 301  CCTTTGCCGA GGGTTCCGC TCTCCGAAGT GCCGTGGGGC GCCGCCTCCC
 351  CCATGCCGCC CTCGGGGCTG CGGCTACTGC CGCTTCTGCT CCCACTCCCG
 401  TGGCTTCTAG TGCTGACGCC CGGGAGGCCA GCCGCGGGAC TCTCCACCTG
 451  CAAGACCATC GACATGGAGC TGGTGAAACG GAAGCGCATC GAAGCCATCC
 501  GTGGCCAGAT CCTGTCCAAA CTAAGGCTCG CCAGTCCCCC AAGCCAGGGG
 551  GAGGTACCGC CCGGCCCGCT GCCCGAGGCG GTGCTCGCTT TGTACAACAG
 601  CACCCGCGAC CGGGTGGCAG GCGAGAGCGC CGACCCAGAG CCGGAGCCCG
 651  AAGCGGACTA CTATGCTAAA GAGGTCACCC GCGTGCTAAT GGTGGACCGC
 701  AACAACGCCA TCTATGAGAA AACCAAAGAC ATCTCACACA GTATATATAT
 751  GTTCTTCAAT ACGTCAGACA TTCGGGAAGC AGTGCCCGAA CCCCCATTGC
 801  TGTCCCGTGC AGAGCTGCGC TTGCAGAGAT TAAAATCAAG TGTGGAGCAA
 851  CATGTGGAAC TCTACCAGAA ATATAGCAAC AATTCCTGGC GTTACCTTGG
 901  TAACGGCTG CTGACCCCCA CTGATACGCC TGAGTGGCTG TCTTTTGACG
 951  TCACTGGAGT TGTACGGCAG TGGCTGAACC AAGGAGACGG AATACAGGGC
1001  TTTCGATTCA GCGCTCACTG CTCTTGTGAC AGCAAAGATA ACAAACTCCA
1051  CGTGGAAATC AACGGGATCA GCCCCAAACG TCGGGGCGAC CTGGGCACCA
1101  TCCATGACAT GAACCGGCCC TTCCTGCTCC TCATGGCCAC CCCCCTGGAA
1151  AGGGCCCAGC ACCTGCACAG CTCACGGCAC CGGAGAGCCC TGGATACCAA
1201  CTATTGCTTC AGCTCCACAG AGAAGAACTG CTGTGTGCGG CAGCTGTACA
1251  TTGACTTTAG GAAGGACCTG GGTTGGAAGT GGATCCACGA GCCCAAGGGC
1301  TACCATGCCA ACTTCTGTCT GGGACCCTGC CCCTATATTT GGAGCCTGGA
1351  CACACAGTAC AGCAAGGTCC TTGCCCTCTA CAACCAACAC AACCCGGGCG
1401  CTTCGGCGTC ACCGTGCTGC GTGCCGCAGG CTTTGGAGCC ACTGCCCATC
1451  GTCTACTACG TGGGTCGCAA GCCCAAGGTG GAGCAGTTGT CCAACATGAT
1501  TGTGCGCTCC TGCAAGTGCA GCTGAAGCCC CGCCCCGCCC CGCCCCTCCC
1551  GGCAGGCCCG GCCCCGCCCC CGCCCCGCC
```

mTGF-β1 PCR Product

```
   1                                                      G
  51  TCACTGGAGT TGTACGGCAG TGGCTGAACC AAGGAGACGG AATACAGGGC
 101  TTTCGATTCA GCGCTCACTG CTCTTGTGAC AGCAAAGATA ACAAACTCCA
 151  CGTGGAAATC AACGGGATCA GCCCCAAACG TCGGGGCGAC CTGGGCACCA
 201  TCCATGACAT GAACCGGCCC TTCCTGCTCC TCATGGCCAC CCCCCTGGAA
 251  AGGGCCCAGC ACCTGCACAG CTCACGGCAC CGGAGAGCCC TGGATACCAA
 301  CTAT
```

Appendix III.

As with the hTGF-β1 RT-PCR product, sample bands were electroeluted and sent for sequencing, this time to ensure that it was endogenous TGF-β1 being detected and was not the action of non-specific binding of the primers. Results of sequencing are shown, and the sequence of the isolated band correctly matches that of mTGF-β1. Probes were made from this band for northern analysis of endogenous TGF-β1 expression after wounding. Single lines indicate the primer annealing site; double lines denote PCR product sequence in relation to TGF-β gene sequence. Primer sequences can be found in Materials and Methods.

Appendix IV—Wound Re-epithelialization

Day 0

	Acutal Numbers		Percentage	
	Open	Closed	Open	Closed
Normal	40	0	100%	0%
Heterozygous	40	0	100%	0%
Homozygous	40	0	100%	0%

Day 5

	Acutal Numbers		Percentage	
	Open	Closed	Open	Closed
Normal	35	5	88%	12%
Heterozygous	36	4	90%	10%
Homozygous	38	2	95%	5%

Day 7

	Acutal Numbers		Percentage	
	Open	Closed	Open	Closed
Normal*	19	13	59%	41%
Heterozygous	29	11	73%	27%
Homozygous	34	6	85%	15%

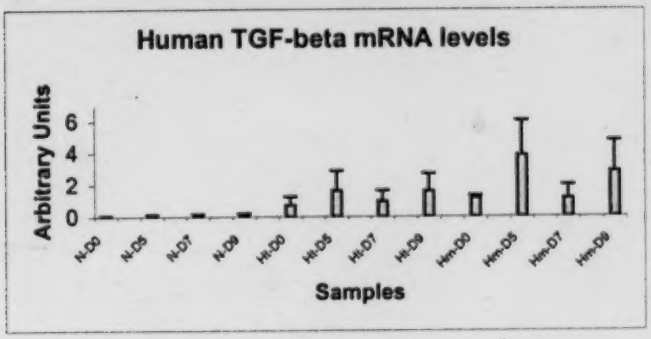
*Note: n=32 for the normal animals at day 7 because two animals (8 wounds) were excluded due to self-aggravation of the wounds which impaired healing.

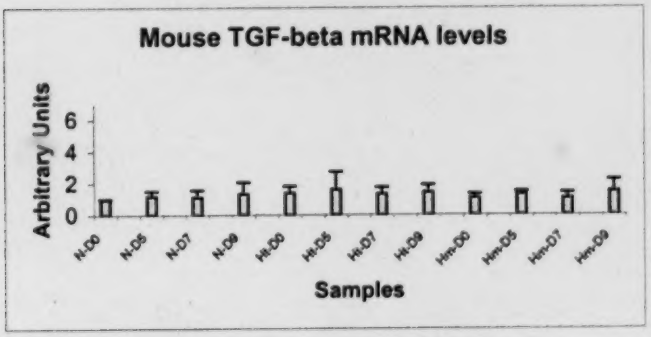
Day 9

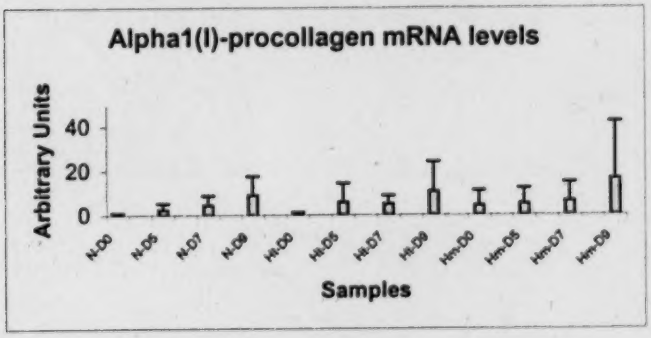
	Actual Numbers		Percentage	
	Open	Closed	Open	Closed
Normal**	7	29	19%	81%
Heterozygous	25	15	63%	37%
Homozygous	22	18	55%	45%

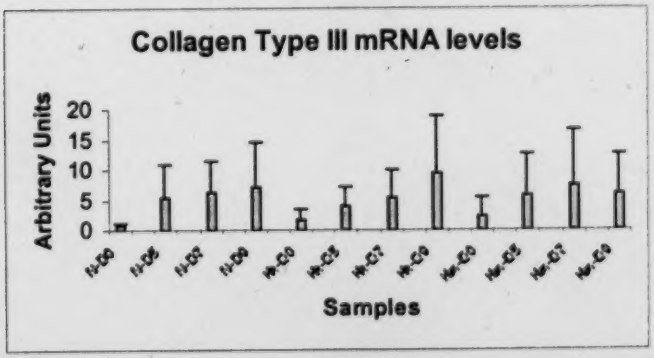
**Note: n=36 for the normal animals at day 9 because one animal (4 wounds) were excluded due to self-aggravation of the wounds which impaired healing.

Appendix V—Scanned Northern Analysis

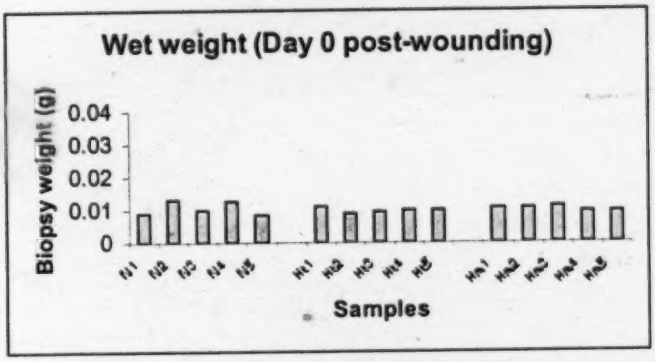


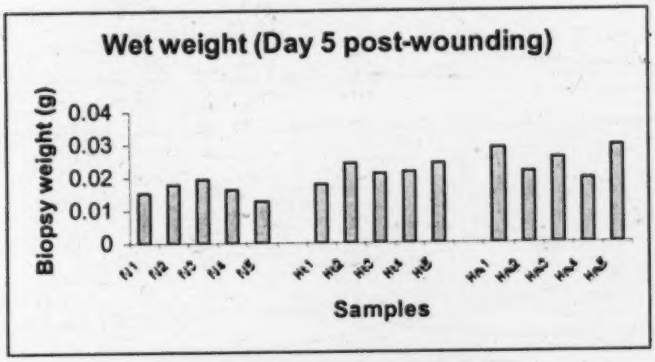


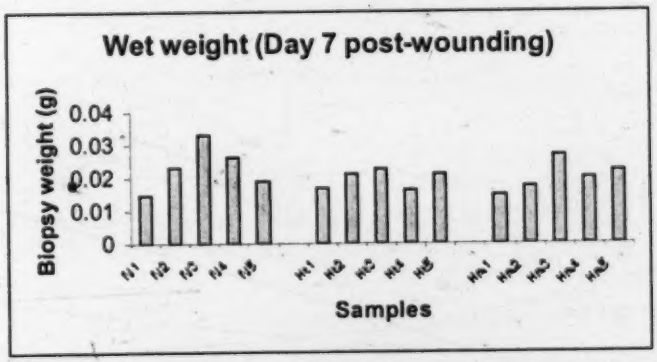


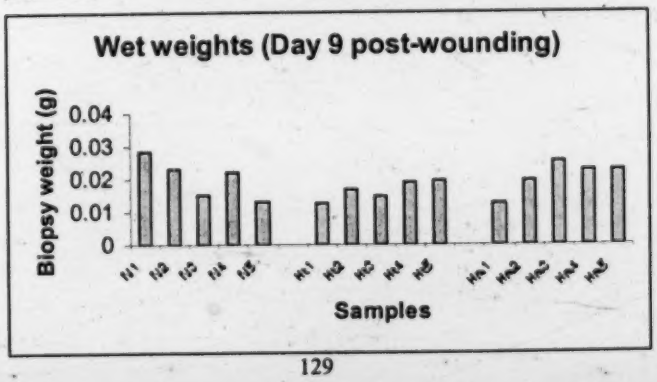


Appendix VI

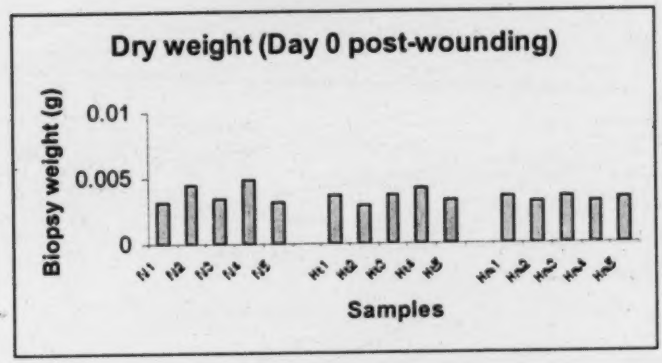

Wet weight (Day 0 post-wounding)
Biopsy weight (g)
0.04
0.03
0.02
0.01
0
Samples

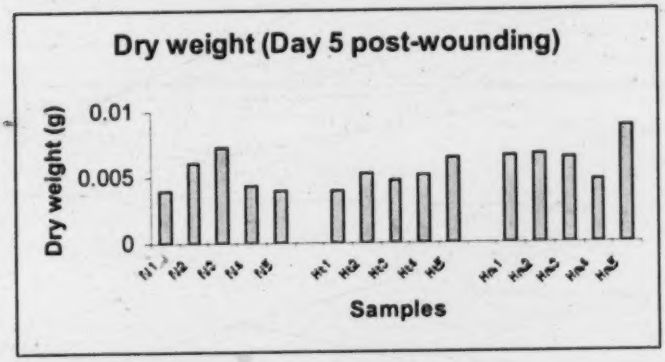

Wet weight (Day 5 post-wounding)
Biopsy weight (g)
0.04
0.03
0.02
0.01
0
Samples

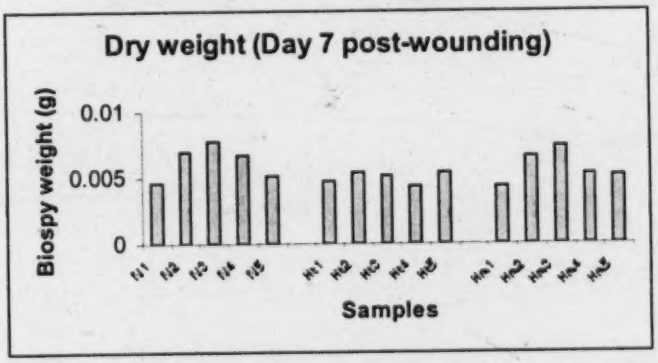

Wet weight (Day 7 post-wounding)
Biopsy weight (g)
0.04
0.03
0.02
0.01
0
Samples

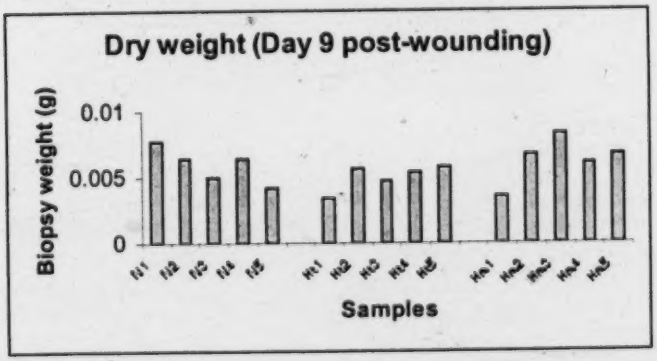

Wet weights (Day 9 post-wounding)
Biopsy weight (g)
0.04
0.03
0.02
0.01
0
Samples

Appendix VII

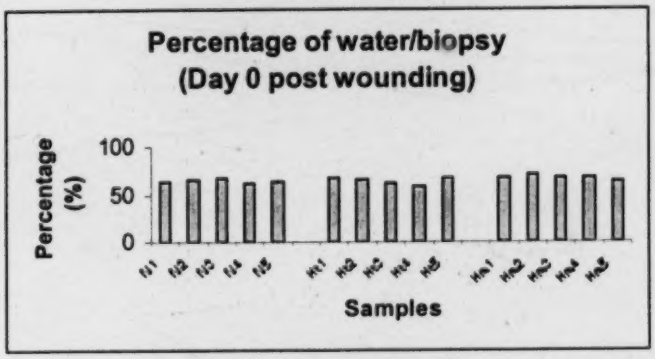


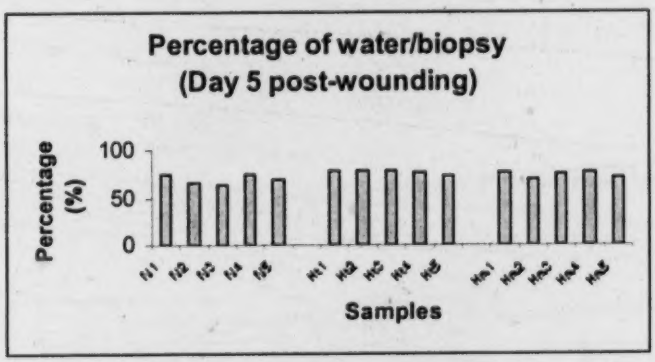


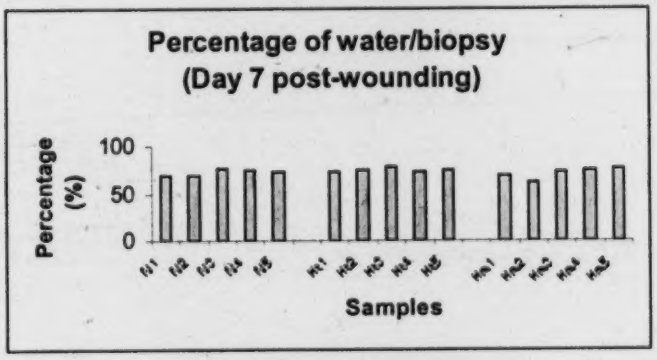


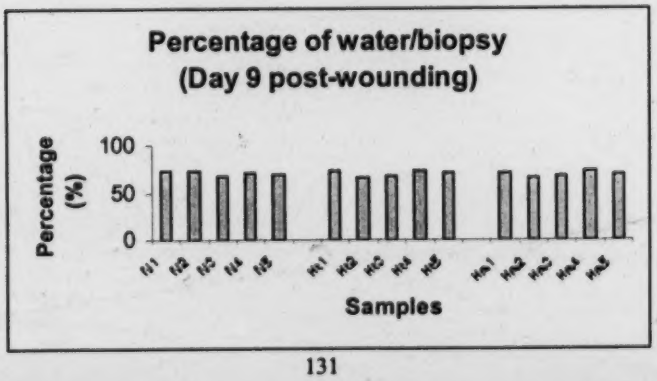


Appendix VIII









Appendix IX—RAW DATA

hTGF-β1 Northern Scans (n=4)

Normal	Day 0	Day 5	Day 7	Day 9
Mean	0.024313	0.051241	0.063869	0.067396
Standard Deviation	0.048626	0.102483	0.127739	0.134793

Heterozygous	Day 0	Day 5	Day 7	Day 9
Mean	0.750001	1.48032	1.258164	1.606426
Standard Deviation	0.5	0.978295	0.712462	0.848972

Homozygous	Day 0	Day 5	Day 7	Day 9
Mean	1.610669	3.838168	1.862848	3.182076
Standard Deviation	1.074862	2.070151	1.632227	2.121728

mTGF-β1 Northern Scans (n=4)

Normal	Day 0	Day 5	Day 7	Day 9
Mean	1.0	1.173483	1.10799	1.338749
Standard Deviation	0.0	0.316359	0.477064	0.719566

Heterozygous	Day 0	Day 5	Day 7	Day 9
Mean	1.391422	1.624174	1.339443	1.441048
Standard Deviation	0.436661	1.090832	0.416344	0.434076

Homozygous	Day 0	Day 5	Day 7	Day 9
Mean	1.069239	1.294537	1.058515	1.458572
Standard Deviation	0.257415	0.183431	0.329576	0.761988

Appendix IX (continued)

α1-Procollagen Northern Scans (n=4)

Normal	Day 0	Day 5	Day 7	Day 9
Mean	1.0	2.465131	4.47126	9.047779
Standard Deviation	0.0	2.892501	4.364028	8.567583

Heterozygous	Day 0	Day 5	Day 7	Day 9
Mean	0.702739	5.627177	5.009425	10.51991
Standard Deviation	0.567189	8.548525	3.833816	13.58877

Homozygous	Day 0	Day 5	Day 7	Day 9
Mean	4.28963	5.000452	6.248502	16.16546
Standard Deviation	6.601144	6.92009	8.387754	25.79282

Type III Collagen Northern Scans (n=2)

Normal	Day 0	Day 5	Day 7	Day 9
Mean	1.0	5.380912	6.164473	7.021744
Standard Deviation	0.0	5.344632	5.382761	7.589293

Heterozygous	Day 0	Day 5	Day 7	Day 9
Mean	1.688371	3.863967	5.351804	9.512194
Standard Deviation	1.798722	3.292565	4.620912	9.298289

Homozygous	Day 0	Day 5	Day 7	Day 9
Mean	2.371076	5.697583	7.487411	5.908788
Standard Deviation	3.166026	6.91411	8.960755	6.545562